TITLE: North American fireflies host low bacterial diversity.

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ABSTRACT

Although there are numerous studies of fireflies' mating flashes, lantern bioluminescence, and anti-predation lucibufagin metabolites, almost nothing is known about their microbiome. We therefore used 16S rRNA community amplicon sequencing to characterize the gut and body microbiomes of four North American firefly species: *Ellychnia corrusca*, *Photuris* sp., *Pyractomena borealis*, and *Pyropyga* sp. These firefly microbiomes all have very low species diversity, often dominated by a single species, and each firefly species has a characteristic microbiome. Although the microbiomes of male and female fireflies did not differ, *Photuris* sp. gut and body microbiomes did, with their gut microbiomes being enriched in *Pseudomonas* and *Acinetobacter*. *E. corrusca* egg and adult microbiomes differed except for a single egg microbiome that shared a community type with *E. corrusca* adults, which could suggest microbial transmission from mother to offspring. Mollicutes that had been previously isolated from fireflies were common in our firefly microbiomes. These results set the stage for further research concerning focus on the function and transmission of these bacterial symbionts.

INTRODUCTION:

Beetles (Order: Coleoptera) are one of the most diverse insect groups, containing nearly 400,000 species (1). The beetles in the family Lampyridae are commonly known by many names: fireflies, lightning bugs, glow worms, lamp-lighters, and night-travelers, all describing the bioluminescent lantern that they use to signal to their mates (2). Fireflies are found on every continent except Antarctica, living in woods, plains, and marshes. North America is home to over 125 firefly species, including *Ellychnia corrusca*, *Photuris* sp., *Pyractomena borealis* and *Pyropyga* species (2). These 4 species occur sympatrically, ranging from the eastern coast of

Canada, south to Florida, and west to the Great Plains (3, 4). *E. corrusca*, *P. borealis* and *Pyropyga* sp. are all closely related to each other, and both *E. corrusca* and *Pyropyga* sp. are diurnal with no lantern (2, 5). *E. corrusca*, *P. borealis*, and many other North American fireflies use lucibufagins as a chemical defense. Lucibufagins are cardioactive C-24 steroidal pyrenes and a subclass of the bufadienolides (6–11) that create a disagreeable taste to predators and thereby provide fireflies with protection from bats, birds and spiders (12, 13). However, the biosynthetic origin of lucibufagins is not known.

In this study, we focus especially on two of these firefly species with unique lifestyles: *E. corrusca* and *Photuris*. *E. corrusca* is nicknamed the winter firefly because it has a winter-spring activity cycle, in contrast to the late spring-summer activity cycles of most other fireflies (1, 3). *E. corrusca* larvae in New England (North America) extend their larval stage across two years instead of one to ensure they ingest ample calories, emerging in the late fall of the second year as adults and sexually maturing during the winter to start mating, which lasts until late spring (14). Normally, adult fireflies do not feed. However, northern (Massachusetts, USA) *E. corrusca* firefly adults ingest interstitial fluid and sap from maple trees, which is thought to help them survive during the cold winters (4, 14)**.** The *Photuris* adult life cycle occurs during the summer months (June-August), but unlike other fireflies, *Photuris* sp. females mimic the mating light display of female *Photinus* fireflies to attract *Photinus* males, earning them the nickname "femme fatale" (15). Once a male *Photinus* firefly is near, the *Photuris* female will attack and kill the male, gaining nutrients and lucibufagins that she uses to protect herself, her eggs, and her pupae, which do not themselves produce lucibufagins (6). Although *E. corrusca* are not active during the same season as *Photuris* sp., *Photuris* sp. will attack and eat *E. corrusca* to gain

protective lucibufagins in the lab (2, 10). This predation in a lab setting could give insight into the selective pressures that caused *E. corrusca* to mate in the winter instead of in the summer.

Beetles feed on many different substrates, following herbivorous (16) omnivorous (17), xylophagous (18, 19), detritivorous (20), and predatory diets (6). Beetles belonging to similar taxonomic families but that have different diets have distinct microbial communities (21, 22). Diets lacking in nutrients often cause insects to rely on nutritional symbionts to provide vital nutrients (23), as in dung (24), carabid (17), and carrion beetles (25). Although there is a vast amount of research studying beetle microbiomes, only a handful of studies consider firefly-associated microbes. In those studies, several Mollicute bacteria were isolated from the fireflies *E. corrusca* and *Photuris* spp., but this does not give insight into the composition of their microbiome beyond these strains (26–30). To fill this gap, we used 16S rRNA community amplicon sequencing to survey the microbiomes of *Photuris* sp., *E. corrusca*, *Pyractomena borealis*, and *Pyropyga* sp. fireflies. Our results show that fireflies have simple, species-specific microbiomes, and generate hypotheses about how diet and seasonality may drive firefly microbiome structure and function.

METHODS

Sample collection

Live *E. corrusca* and *Photuris* sp. fireflies were collected by S. Smedley during the winter of 2016-17 and spring/summer of 2017 from residential and camping/forest areas within Vernon, Bolton and Andover, Connecticut, U.S.A. *Pyractomena borealis* larvae and adults were raised in the laboratory by S. Smedley. After collection, *E. corrusca*, *Photuris* sp., and *P. borealis* fireflies were stored at -80°C in air-filled vials. *Pyropyga* sp. fireflies were collected by

Lynn Faust in summer 2016 from Ohio and Tennessee and stored in 95% ethanol. Collection dates and sample locations are listed in Supplemental File 1.

Sample preparation & DNA extraction

All fireflies were surface-sterilized using 3 rounds of a 10 second submersion in 70% ethanol, followed by a 10 second submersion in phosphate-buffered saline (PBS) (31). Surface-sterilized fireflies were dissected into two separate tissues: a gut sample and the remaining carcass "body" sample. DNA from the tissue dissections were extracted using a bead beating and chloroform-isopropanol protocol as described in (32), except using 0.7 g of 1 mm and 0.3 g of 0.1 mm silica/zirconium beads and 5 cycles of bead-beating & chilling on ice. Negative controls containing only the DNA extraction reagents were processed alongside each batch of tissue samples. The DNA concentration of each extract and negative control was determined using the Qubit dsDNA high-sensitivity assay protocol and a Qubit 3.0 fluorimeter (Invitrogen, Carlsbad California).

16S V4 rRNA PCR screen & community amplicon sequencing

DNA samples were PCR amplified using primers 515F and 806R (targeting the bacterial 16S rRNA gene V4 region) to determine the presence of bacterial DNA (33). Ten nanograms of template DNA was added to 5 µl Green GoTaq Reaction Mix Buffer (Promega, Madison, Wisconsin, USA), 1.25 units of GoTaq DNA Polymerase (Promega, Madison, Wisconsin, USA), 10 µmol of each primer, and 300 ng/µl BSA (New England BioLabs Inc. Ipswitch Massachusetts), to which nuclease free H_2O was added to a volume of 25 µl. Thermocycling conditions (BioRad, Hercules, California) were: 3 min at 95°C, 30 cycles of 30 sec at 95°C, 30 sec at 50°C, and 60 sec at 72°C, followed by a 5 min cycle at 72°C and then an indefinite hold at 4°C. Gel electrophoresis was used to confirm the expected band size of 300–350 bp.

All samples that had a gel band of the expected size were prepared for community amplicon sequencing of the 16S rRNA gene V4 region using an Illumina MiSeq at the University of Connecticut Microbial Analysis, Resources and Services (MARS) facility. Approximately 30 ng of DNA from each sample was added to a 96-well plate containing 10 µmol each of the forward and reverse Illumina-barcoded versions of primers 515F and 806R, 5 µl AccuPrime buffer (Invitrogen, Carlsbad, California), 50 mM $MgSO_4$ (Invitrogen, Carlsbad, California), 300 ng/µl BSA (New England BioLabs Inc. Ipswitch, Massachusetts), a 1µmol spike-in of both non-barcoded primers 515F and 806R, and 1 unit AccuPrime polymerase (Invitrogen, Carlsbad, California), to which nuclease-free H_2O was added to a volume of 50 µl. Reaction mixes were separated in triplicate reactions (each with a volume of 16.7 µl) into a 384 well plate using an epMotion 5075 liquid handling robot (Eppendorf, Hamburg, Germany). The resulting 384 well plate was then transferred to a thermocycler (Eppendorf, Hamburg, Germany), which used the following conditions: 2 min at 95°C, 30 cycles of 15 sec at 95°C, 60 sec at 55°C, and 60 sec at 68°C, followed by a final extension for 5 min at 68°C and then an indefinite hold at 4°C. After PCR, triplicate reactions were re-pooled using the epMotion, and DNA concentrations were quantified using a QIAxcel Advanced capillary electrophoresis system (QIAgen, Hilden, Germany). Samples that had concentrations >0.5 ng/µl were pooled using equal weights of DNA to create the final sequencing libraries. Libraries were then bead-cleaned using Mag-Bind RXNPure plus beads (OMEGA, Norcross, Georgia) in a 1:0.8 ratio of sequencing library to bead volume. Cleaned library pools were adjusted to a concentration of 1.1 ng/µl ± 0.1 ng/µl, which was confirmed using the Qubit dsDNA high-sensitivity assay on a Qubit 3.0 fluorimeter (Invitrogen, Carlsbad, California). Microbial community sequencing on an Illumina MiSeq

(Illumina, San Diego, California) was completed in 2 batches, the first composed of 52 samples and the second composed of 195 samples.

Post-sequencing & bioinformatic analyses:

Our DNA sequencing produced 245 16S rRNA community amplicon sequencing datasets, including 22 negative controls. Reads were analyzed using R v3.5.3 (34) and the dada2 v1.11.1 (35) pipeline for amplicon sequence variants (ASVs) (https://benjjneb.github.io/dada2/tutorial.html, accessed: November 11, 2017). Read counts ranged from 2 to 888,868 (Suppl. File S1). Metadata files for the samples were imported into phyloseq v1.26.1 (31, 32), creating a phyloseq R object that was used for subsequent analyses. Reads that were not classified as belonging to the kingdom Bacteria using the SILVA database v128 were removed (38, 39). ASVs that matched to mitochondria were then removed separately, because SILVA included them in the kingdom Bacteria. Samples were screened for contamination using the decontam v1.2.1 (40) prevalence protocol with a default threshold value of 0.1. No reads were flagged as contamination, and 17 samples with 0 reads were removed, resulting in 1,675 unique ASVs (Supplemental File S1). Negative control samples were not considered further. All samples in the dataset were then rarefied to 10,000 reads and read counts were converted to relative abundances. The final phyloseq object contained 133 samples from 20 *Photuris* adults, 4 *Pyropyga* adults, 7 *Pyractomena borealis* larvae, 1 *Pyractomena borealis* adult, 95 *Ellychnia corrusca* adults, and 6 *E. corrusca* eggs. Despite amplification during the initial PCR screen, of the initial 223 insect samples, 74 samples were removed due to low read counts (53 *E. corrusca* adult samples, 3 *E. corrusca* egg samples, 9 *Photuris* sp. samples, 2 *Pyropyga* sp. samples, 4 *P. borealis* adult samples & 3 *P. borealis* larvae samples). These low read counts may have been due to: 1) high amounts of host DNA acting as a PCR inhibitor; 2)

there being a minimal firefly microbiome, leading to limited template concentrations; or 3) other technical issues such as inefficient PCR amplification using primers that contained the Illumina barcodes compared to our initial PCR screen using non-barcoded primers.

Alpha diversity was measured using the phyloseq 'plot_richness' command, and Beta diversity was measured using weighted and unweighted unifrac distance metrics. Weighted and unweighted unifrac distances were calculated, ordinated, and viewed using the 'distance', 'ordinate', and 'plot_ordinate' phyloseq commands, respectively. PERMANOVA statistical tests were calculated using vegan v 2.5-4 (41). Although weighted unifrac (WUF) and unweighted unifrac (UUF) distances were used for each test, to keep the text concise only one test is listed in the text and the complimentary values are presented in Supplementary Tables S1-S4.

Differences in the relative abundances of taxa in firefly microbiomes were compared using DESeq2 (42), using the Parametric fitType, Wald tests, and an adjusted alpha value of 0.01. A heatmap was constructed for the 26 most abundant genera in the dataset using the gplots v3.03 (https://www.rdocumentation.org/packages/gplots) command 'heatmap.2' and using the Euclidean and ward.D distance metrics. To avoid redundancy in the heatmap, ASVs were grouped by genus name using the phyloseq command 'tax_glom'. Covariance between the top 26 taxa were calculated using SpiecEasi v1.0.7 (43), and the SpiecEasi output file was exported into Cytoscape v3.7.2 (44) using igraph v1.2.4.2 (45) for visualization.

The most abundant Mollicute ASVs from our dataset included four *Mesoplasma* ASVs, two *Spiroplasma* ASVs, and one *Entomoplasma* ASV. A phylogenetic tree was constructed to show the relationships between the 16S rRNA sequences of Mollicutes from this study and those that had been previously isolated from fireflies. Reference Mollicute sequences from taxa belonging to the same genera as our firefly ASVs were selected from the SILVA database,

174 especially those Mollicutes that had been isolated from fireflies and other beetles. Additional
175 Mollicute reference sequences for fireflies that were not represented in the SILVA database were
176 downloaded from NCBI. Sequences were aligned using MUSCLE v3.8.31 (46) and trimmed to
177 the same length. The phylogenetic tree, rooted by the 16S rRNA genes from *Bacillus subtilis* and
178 *Mycoplasma haemominutum*, was calculated using a GTRGAMMAI substitution model and 500
179 bootstrap replicates in RAxML v8.2.11 (47). The topology of the tree created using these
180 selected sequences agreed with that constructed using all Mollicute sequences in SILVA,
181 ensuring that taxon selection did not bias our phylogenetic analysis.
182 The commands used for all analyses is attached as Suppl. File S2. All data are available
183 on NCBI under BioProject PRJNA563849. Raw sequencing reads are deposited in SRA under
184 BioSample numbers SAMN14678004 – SAMN14678257.
185
186 **RESULTS**
187 **Firefly microbiomes are typically dominated by single taxa**
188 We characterized the bacterial communities in 133 firefly gut and body dissections
189 (Suppl. File S1) using community amplicon sequencing of the 16S rRNA gene. All firefly
190 microbiomes had low α-diversity (Suppl. Fig. S1). Shannon diversity scores for the firefly
191 microbiomes ranged from 0.70 to 2.52, and alpha diversities of *Photuris* sp. and *E. corrusca*
192 microbiomes differed from each other, as did those from *Pyropyga* sp. and *E. corrusca* (Dunn
193 test, $p = 0.001$ and $p = 0.003$ respectively). A heatmap of the top 26 bacteria genera found in the
194 microbiomes reflects these low levels of α-diversity, with most firefly microbiomes dominated
195 by a single taxon but with minute amounts of other taxa also present (Fig. 1). Unsupervised
196 clustering of these data grouped *E. corrusca* samples tightly together at the left side of the heat

map, while the samples from other species clustered together on the right. Samples from different tissue dissections and sexes did not cluster together for any species of firefly.

Using these top 26 bacteria genera, we identified taxa whose relative abundances were correlated with each other to infer potential interactions between them (Suppl. Fig. S2). Of the 26 bacteria genera, the relative abundances of 14 genera did not correlate with those of another genus in our dataset, and the relative abundances of 11 genera were positively correlated with those of another genus. The relative abundances of *Ralstonia* and *Cupriavidus*, found in the *Pyropyga* sp. samples, were strongly and positively correlated with each other, as were the relative abundances of *Tanticharoenia* and *Gluconobacter*, found in *E. corrusca* eggs, and the relative abundances of *Gordonia* and *Tsukamurella*, found in *Photuris* sp.. Only the relative abundances of *Mesoplasma* and *Acinetobacter* were negatively correlated with each other, and these taxa were not found together in any sample.

In the heatmap, many samples clustered together that were dominated by single taxa. Based on these clusters, we defined community types based on the genus or genera of bacteria that were present in these samples with relative abundances ≥ 30% (Fig. 2). *E. corrusca* adults were assigned to 6 distinct community types: T-1 (*Mesoplasma*), T-2 (*Salmonella*), T-3 (*Mesoplasma* & *Salmonella*), T-4 (*Serratia*), T-5 (*Rickettsia*) and T-6 (*Pseudomonas*). *E. corrusca* egg microbiomes were all assigned to T-13 (*Gluconobacter*), except for a single egg microbiome that belonged to T-2 (*Salmonella)*, the same as some *E. corrusca* adult microbiomes. *Photuris* sp. microbiomes were assigned to 3 community types: T-8 (*Acinetobacter*), T-9 (*Yersinia*) and T-6 (*Pseudomonas*). *Pyractomena borealis* and *Pyropyga* sp. were assigned to T-10 (*Empedobacter*) and T-12 (*Cupriavidus*), respectively.

Firefly species harbor unique microbial communities

The four sampled firefly species had distinct bacterial communities (Weighted Unifrac (WUF) PERMANOVA: R^2=0.215, p=0.001; Fig. 3A, Suppl. Fig. S3, Suppl. Table S1). In a PCoA ordination of WUF beta-diversity distances, *E. corrusca* samples clustered together, spanning from left to right (Fig. 3). *Pyropyga* sp. samples clustered together with some *E. corrusca* samples and those from *Photuris* sp. on the left side of Figure 3A, and *Photuris* sp. samples grouped together down the left side and at the bottom, along with the *Pyractomena borealis* samples. Species-specific clustering can be seen in the UUF plot, with *E. corrusca* having the most variance (Suppl. Fig. S3B). The separation between the *E. corrusca* and *Photuris* sp. microbiomes was maintained in a better-balanced comparison when the few *Pyropyga* sp. and *P. borealis* samples were excluded (WUF PERMANOVA: R^2=0.164, p=0.001; Suppl. Fig S4, Suppl. Table S2).

We identified the bacterial taxa whose relative abundance differed between *E. corrusca* and *Photuris* sp. fireflies using DESeq. Although *Pseudomonas* ASVs were found in both fireflies, one *Pseudomonas* ASV was more abundant in *Photuris* sp. and two other *Pseudomonas* ASVs were more abundant in *E. corrusca* (Fig. 3B). Two *Mesoplasma* ASVs, *Salmonella*, *Serratia*, *Rickettsia* and *Rickettsiella* were all more abundant in *E. corrusca* than in *Photuris* sp., and *Tsukamurella*, *Gordonia*, *Curvibacter* and *Sphingobacterium* were all more abundant in *Photuris* sp. than in *E. corrusca* (Fig. 3B).

Gut and body microbiomes are distinct, but these differences are firefly-specific

The above analysis indicated that the four sampled firefly species all hosted distinct microbiomes (Fig. 3). However, further analyses using these same data also indicated that different firefly tissues might also host distinct microbiomes, in a species-specific manner (WUF PERMANOVA for the interactions between species and tissue: R^2=0.048, p=0.003; Suppl. Fig.

S3, Suppl. Table S1). In contrast, a parallel analysis using only samples for which sex was determined indicated that microbiomes did not differ between the sexes, regardless of species or tissue type (all PERMANOVAs testing for differences between sexes: $p > 0.05$; Suppl. Fig. S5, Suppl. Table S3). In the analysis described above with *Pyractomena borealis* and *Pyropyga* sp. samples removed to avoid possible artifacts due to unbalanced sample sizes, firefly tissue microbiomes again differed in a species-specific manner (WUF PERMAONVA for interactions between *E. corrusca* and *Photuris* sp. gut and body microbiomes: $R^2=0.053$, $p=0.001$; Suppl. Fig. S4, Suppl. Table S2).

Because these PERMANOVAs indicated that tissue microbiomes differed in a species-specific manner, we repeated our analysis for each species separately. *E. corrusca* eggs, gut and body microbiomes differed from each other (WUF PERMANOVA: $R^2=0.122$, $p=0.001$), with most gut and body samples clustered together in the PCoA and most egg samples clustered separately (Fig. 4A, Suppl. Table S4, Suppl. Fig. S6). When the egg samples were removed from the analysis, *E. corrusca* gut and body microbiomes differed from each other only when using the UUF distance metric (UUF PERMANOVA: $R^2= 0.072$, $p=0.001$; WUF: Suppl. Table S4). *Wolbachia* had low relative abundance in all *E. corrusca* samples, but was the only bacterial genus that more abundant in *E. corrusca* eggs than in adults. *Mesoplasma*, *Pseudomonas*, *Acinetobacter* and several other genera were all more abundant in *E. corrusca* adults than in eggs (Fig. 4B). *E. corrusca* eggs and adults also had distinct community types, with *E. corrusca* egg microbiomes mainly assigned to T-13 (*Gluconobacter*), and only a single egg microbiome assigned to T-3 (*Salmonella*) like those of *E. corrusca* adults (Fig. 2). In contrast, *Photuris* sp. gut and body microbiomes more strongly differed from each other (WUF PERMANOVA: $R^2= 0.340$, $p=0.001$; Fig. 4C, Suppl. Fig. S7, Suppl. Table S4), with *Pseudomonas*, *Acinetobacter*,

Leucobacter, *Elizabethkingia*, *Curvibacter* and *Achromobacter* all being more abundant in *Photuris* sp. guts than in bodies (Fig. 4D). *Pyropyga* sp. gut and body microbiomes did not differ from each other (WUF PERMANOVA: R^2=0.312, p=0.667; Suppl. Table S4), and *P. borealis* gut and body microbiomes differed only when using the UUF distance metric (UUF PEMANOVA: R^2= 0.616, p=0.008; Suppl. Table S4). These conclusions should be considered preliminary because of the small sample sizes available for both *Pyropyga* sp. and *P. borealis*.

Fireflies host an abundance of potentially symbiotic Mollicutes

Mollicutes were the most abundant bacteria in our 133 firefly samples, consistent with the handful of Mollicutes that had previously been isolated from different firefly species (27, 29, 30, 48–50). We therefore created a phylogenetic tree to discover how our most abundant Mollicute ASVs were related to the sequences of these known firefly Mollicute isolates (Fig. 5). The most prevalent *Spiroplasma* ASV (ASV Spiroplasma1), found in many of the *E. corrusca* samples, was similar to the 16S rRNA sequence of *S. corruscae*, a species that was first isolated from *E. corrusca* (28). ASV Spiroplasma2 was found in one *Photuris* sp. sample, and was similar to the 16S rRNA sequence of *S. ixodetis*, a male-killing agent in butterflies (51). *S. ixodetis* has not been found in fireflies to date. The four *Mesoplasma* ASVs, mainly found in *E. corrusca*, are all similar to the 16S rRNA sequences for *M. corruscae* and *Entomoplasma ellychniae*, which had both been isolated previously from *E. corrusca* (29, 48, 49). Although originally separated using cell morphology and culture media requirements, the genera *Mesoplasma* and *Entomoplasma* are paraphyletic (52). Our phylogenetic tree showing that both *M. corruscae* and *E. ellychniae* cluster tightly together suggests that *E. ellychniae* should be reclassified as a member of the genus *Mesoplasma*. The single *Entomoplasma* ASV, found in *Pyractomena borealis* adults, closely resembled the 16S rRNA sequence for *E. somnilux*, which

had been previously isolated from *Pyractomena angulate* (27). This phylogenetic tree shows that the Mollicutes sequences detected in study are highly similar to strains that had been previously isolated from fireflies, except for that related to *S. ixodetis*.

DISCUSSION:

Our results show that all of our sampled species of firefly have low-complexity microbiomes. The alpha diversity of our firefly microbiomes was very low, and all samples had only a few taxa present in high abundance (Fig 1). The low number of correlations between the relative abundances of firefly taxa (Suppl Fig. S2) is consistent with this predominance of simple microbiomes, because (by definition) few interactions can occur when only a single bacterium is abundant in a microbiome, and correlations will inevitably be weak when there is high variability between microbial communities. This variance and low diversity could be explained by microbiomes being acquired via neutral community assembly. Fireflies may be exposed to environmental bacteria by chance, and these bacteria may then multiply and form a stable microbiome. This would explain why there is high variance between the microbiomes from individual insects of the same firefly species.

Samples could be assigned to one of thirteen community types, defined by the taxa in these samples with >30% abundance. Different firefly species rarely shared the same community type, and most community types were defined by a single taxon, with only one community type having two taxa that were each >30% abundant (Fig. 2). The presence of multiple community types in each firefly species creates questions about whether the bacteria are either: 1) resident members replicating within the firefly, or 2) transient members that replicate more slowly than the rate of expulsion from the firefly (53). Although such transient microbiomes are not likely to be conserved between fireflies, it is possible that they do have some effect on their hosts

312 (positive or negative). Community types vary within the same firefly species, suggesting that
313 these microbiomes are transient, some bacteria were found in extremely high relative abundances
314 (Fig. 1) that could suggest a stable resident microbiome of unknown function. It is possible that
315 each community type provides a different function for the firefly. It is also possible that the
316 multiple, highly abundant bacteria in different community types, such as *Mesoplasma*,
317 *Salmonella*, and *Pseudomonas*, could provide similar functions for their hosts, although this may
318 be unlikely for Mollicute bacteria due to their reduced genomes.

319 Fireflies from different species also had distinct microbiomes (Fig. 3A). *E. corrusca* and
320 *Photuris* sp. had distinct bacterial communities, and samples from the same species clustered
321 together in PCoA plots of beta-diversity (Suppl. Fig. 4). Differences between the microbiomes of
322 these two species might be due to their different lifestyles. North American *E. corrusca* are
323 active in winter and may feed on tree sap (4, 14), and so *E. corrusca* microbial communities may
324 therefore be acquired via the ingestion of these fluids. Unlike *E. corrusca*, *Photuris* sp. are
325 predatory and active in the summer. Insect gut microbiomes often vary based on differences in
326 their host's habitat and diet (e.g., omnivory vs. carnivory) (22), and such differences might
327 underlie the differences that we observed between *E. corrusca* and *Photuris* sp. microbiomes.
328 Both *E. corrusca* and *Photuris* sp. hosted *Pseudomonas* ASVs that were similar to those
329 previously found on trees and in soils, which could be explained by *E. corrusca* and *Photuris* sp.
330 living in soil, on tree bark, and on plant leaves (2), where *Pseudomonas* is common (54). All of
331 the *E. corrusca* and *Photuris* sp. samples used in this study were collected from Connecticut,
332 USA, and whether these microbiomes vary in other geographic locations needs future research.

333 For some firefly species, eggs, guts, and bodies had distinct microbiomes. *E. corrusca*
334 adult and egg microbiomes differed from each other, with samples clustering separately in

335 PCoAs (Fig. 4A, Suppl. Fig. S6), but an *E. corrusca* egg also had the same community type (T-
336 2; *Salmonella*) as that of some adults. Although it is unknown which firefly laid this egg, these
337 results suggest that it might be possible for *Salmonella* to be transmitted vertically between *E.*
338 *corrusca* adults and eggs. The mechanisms that *E. corrusca* may use to acquire these microbes
339 from the environment are unknown, but there are other insects that rely on horizontal
340 transmission for their symbionts, such as the bean bug *Riptortus pedestris*, which acquires its
341 *Burkholderia* symbiont from soil each generation using a mucus-filled organ that the
342 *Burkholderia* specifically penetrates and colonizes (55–57). Although *E. corrusca* is not known
343 to have an organ that selects for a specific bacterial symbiont, *Mesoplasma* was found in many
344 samples of *E. corrusca*. It is possible that *E. corrusca* may select this bacterium from the
345 environment, or that *Mesoplasma* selects *E. corrusca* as a preferred host. *E. corrusca* is the most
346 sampled firefly species in our study, and hosts microbiomes belonging to the most community
347 types. This increased sampling may therefore have increased the number of community types
348 detected in this organism relative to the other species in our study.

349 *Photuris* sp. gut and body microbiomes also differed from each other, with gut
350 microbiomes having higher amounts of *Pseudomonas* and *Acinetobacter* than body microbiomes
351 (Figs. 4C and 4D). These ASVs may be acquired from the firefly's environment, because both
352 ASVs were similar to other strains of bacteria that are common in soil and on plants. The
353 bacteria present in the *Photuris* sp. gut may be derived from eating other fireflies, and thus the
354 *Pseudomonas* ASVs that are common in *Photuris* sp. guts may originate from their last meal.
355 The *Photuris* microbiome may therefore be transient and composed of the prey's microbiome.
356 Alternatively, it is possible that the *Photuris* sp. gut microbiome is not transient, and the gut
357 provides a niche that is favorable for the growth of *Pseudomonas* and *Acinetobacter* microbes

that might originate from soil, plants, or the prey's microbiome and subsequently colonize the *Photuris* sp. gut. The origin of the body microbiome remains unclear. Body dissections include all other parts of the firefly except for the gut, meaning that other non-gut tissues and hemolymph from inside of the firefly could have been colonized with bacteria. Alternatively, adult firefly abdomens are surrounded with sclerotized segments, called tergites, on their ventral and dorsal sides (2). These tergites have small gaps between them that allow for movement. Although our firefly samples were ethanol-washed before dissection, this sterilization may not be absolutely perfect, and the body microbiome may therefore include bacteria that are trapped within these segments (58). The bacteria trapped between these tergites may be acquired during constant movement on tree bark or in the leaf litter, where many bacteria are found.

Mollicutes have been isolated from several different firefly species, but their prevalence and function is unclear (26, 27, 50, 59, 60). We detected 4 *Mesoplasma* ASVs in North American fireflies, mainly in *E. corrusca*, that all closely resembled *M. corruscae* and *Entomoplasma ellychniae*, which were both isolated previously from *E. corrusca*. As mentioned in the results, *M. corruscae* and *E. ellychniae* very likely both belong to genus *Mesoplasma* (52). *Mesoplasma* frequently occur on plants without causing disease, and therefore it could be horizontally acquired by *E. corrusca* when they feed on nutrient-poor tree sap to aid in survival during cold winter months (4, 14).. Future work will be required to determine the function, if any, of these Mollicutes in their firefly hosts.

Our results provide the first description of the microbiomes found in *E. corrusca*, *Photuris* sp., *Pyropyga* sp. and *Pyractomena borealis*. These microbiomes have low alpha diversities and are species-specific. Some firefly species also have distinct egg, gut, and body

380 microbiomes. Future research will determine the function of these microbiomes and how they are
381 acquired from their environment or transmitted between hosts.
382
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392

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565

FIGURE LEGENDS

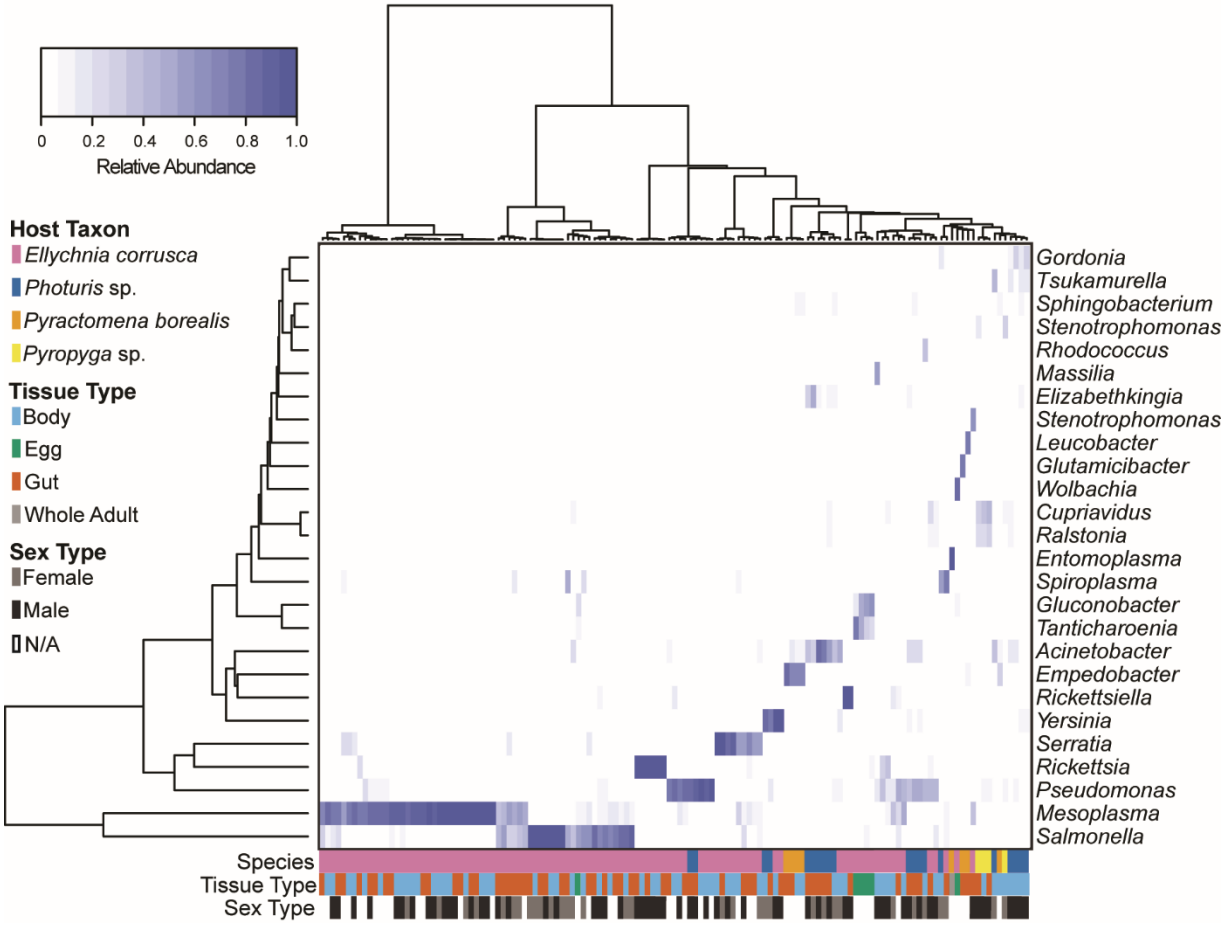
Figure 1) Heat map of the 26 most abundant bacterial genera found in the studied fireflies. To the left, a dendrogram represents the Euclidian distances between the relative abundances of reads assigned to each bacterial genus, which are labeled on the right. The top dendrogram clusters the relative abundances of these genera in each firefly sample using ward.D distances. On the bottom, 3 different rows indicate the host taxon, tissue type, and sex type for each sample, indicated by the different colors in the key to the left of the figure. The topmost color key represents the relative abundance of reads in each sample that were assigned to each bacterial genus, with white and dark blue representing 0 and 100% relative abundance, respectively. n = 133

Figure 2) Firefly microbial community types. The X axis indicates the number of samples that were assigned to each community type. Colors indicate firefly species, as indicated by the key. Note that some gut and body samples originate from the same individual firefly and so are counted twice.

Figure 3) A: PCoA of Weighted Unifrac distances between microbial communities for all four firefly species. Species are differentiated using colors. n = 133. B: Over- and underrepresented ASV sequences in *E. corrusca* and *Photuris* sp. adults, including both gut and body samples. The X-axis indicates the genus of bacteria whose relative abundances differed between firefly species, and the Y-axis indicates the log2 fold change in relative abundance between samples, where the higher numbers indicate overrepresentation in *Photuris* and negative numbers indicate overrepresentation in *E. corrusca*. Colors indicate phyla. n = 114

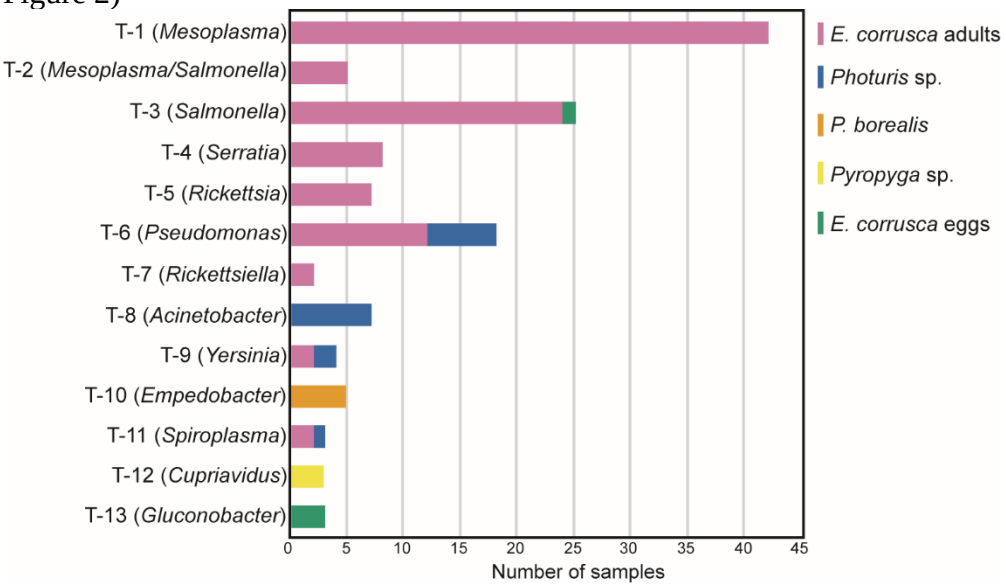
588 Figure 4) A) PCoA of Weighted Unifrac distances between microbiomes of *E. corrusca* eggs and
589 adults. Egg, gut and body samples are differentiated using colors. n = 100. B) Over- and
590 underrepresented ASV sequences in *E. corrusca* egg and adult samples. The X-axis indicates the
591 genera of bacteria whose relative abundance differed between *E. corrusca* eggs and adults, and
592 the Y-axis indicates the log2 fold change in these relative abundances between tissues, where the
593 higher numbers indicate overrepresentation in eggs and negative numbers indicate
594 overrepresentation in adults. Colors indicate phyla. C) PCoA of Weighted Unifrac distances
595 between microbiomes of *Photuris* sp. gut and body samples. Gut and body samples are
596 differentiated using colors. D) Over- and underrepresented ASV sequences of *Photuris* sp. gut
597 and body samples. The X-axis indicates the genus of bacteria whose relative abundance differed
598 between *Photuris* sp. gut and body samples, and the Y-axis indicates the log2 fold change in
599 these relative abundances between tissues, where the higher numbers indicate an
600 overrepresentation in the body samples. Colors indicate phyla. n = 20
601 Figure 5) Phylogenetic tree of the Mollicute 16S rRNA gene ASVs in our dataset (in bold) and
602 reference 16S rRNA gene sequences, particularly those isolated from fireflies and beetles
603 (indicated by a ^). The tree was constructed using RAxML with 500 bootstraps and rooted using
604 *Bacillus subtilis* and *Mycoplasma haemominutum*. Bootstrap values of 60–79 and 80–100 are
605 indicated by * and **, respectively. Numbers in parentheses indicate the number of samples in
606 which that ASV was found, and colors group sequences from the same genera. NCBI accession
607 numbers are shown to the right of each reference sequence. *Me = Mesoplasma, My =*
608 *Mycoplasma*.
609

610 Figure 1)



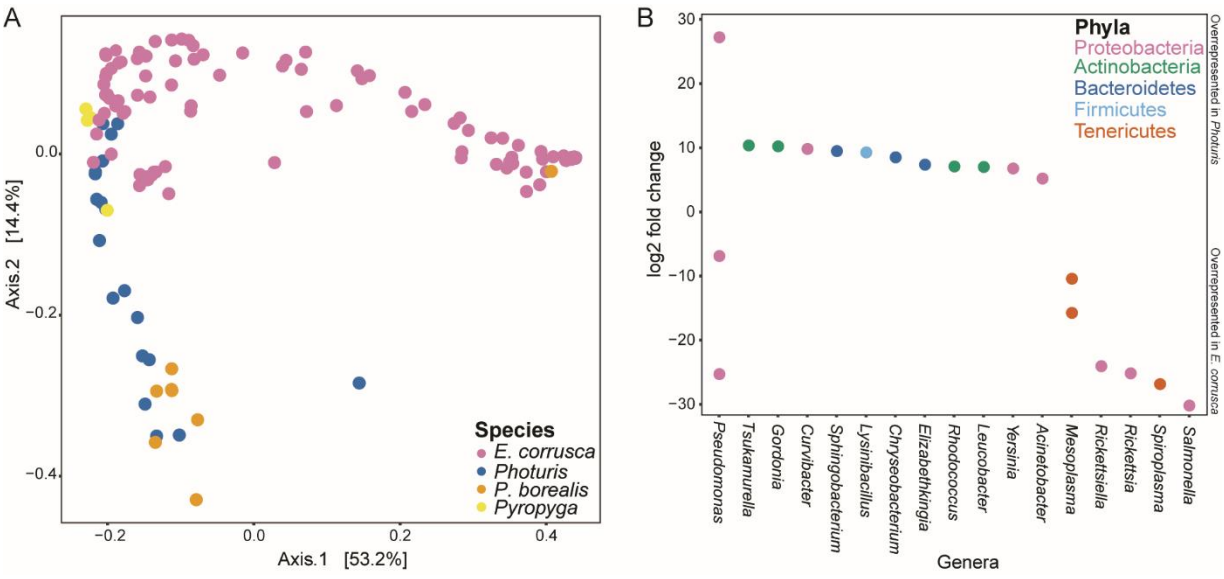
611

612 Figure 2)



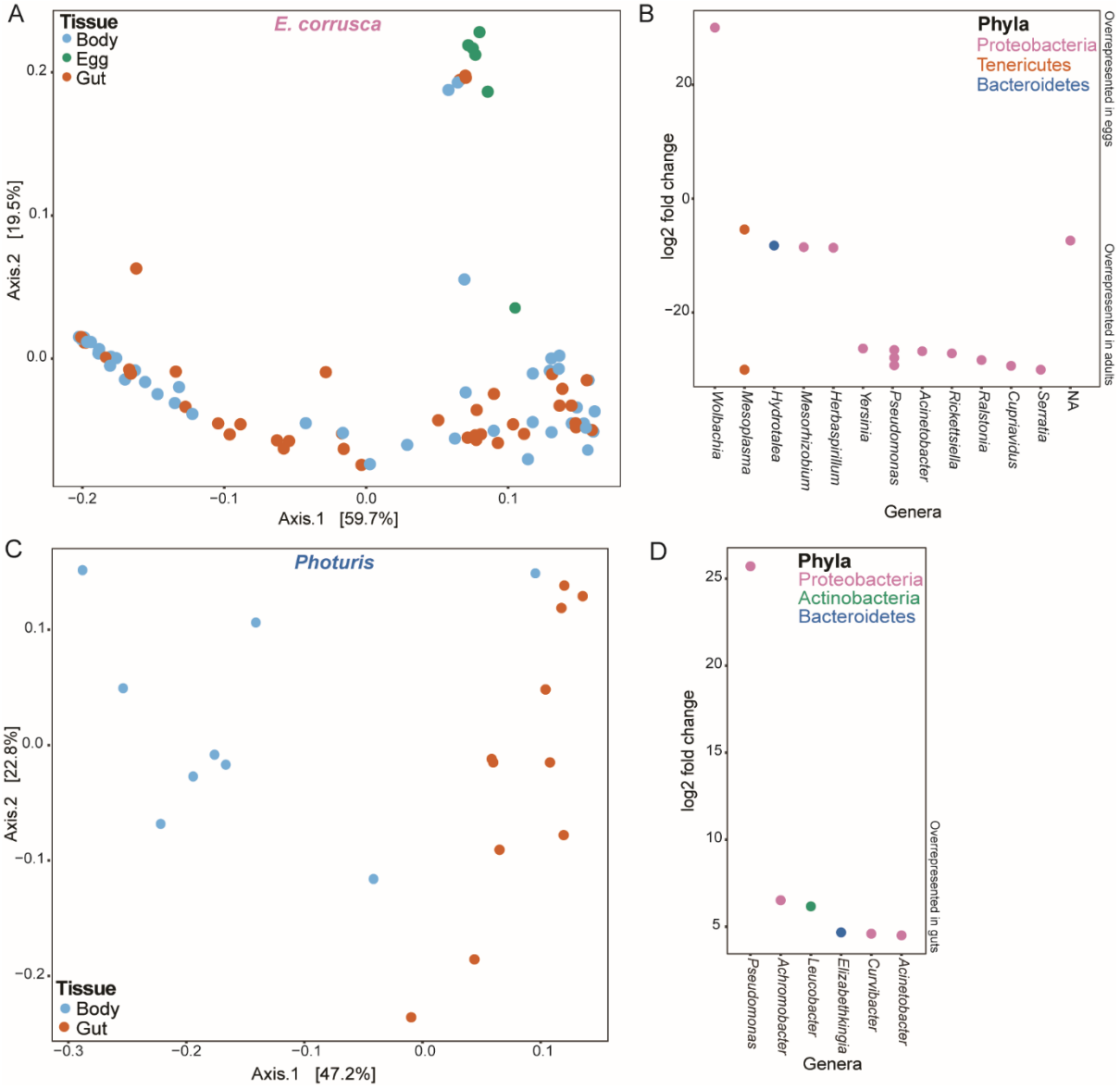
613

614 Figure 3)

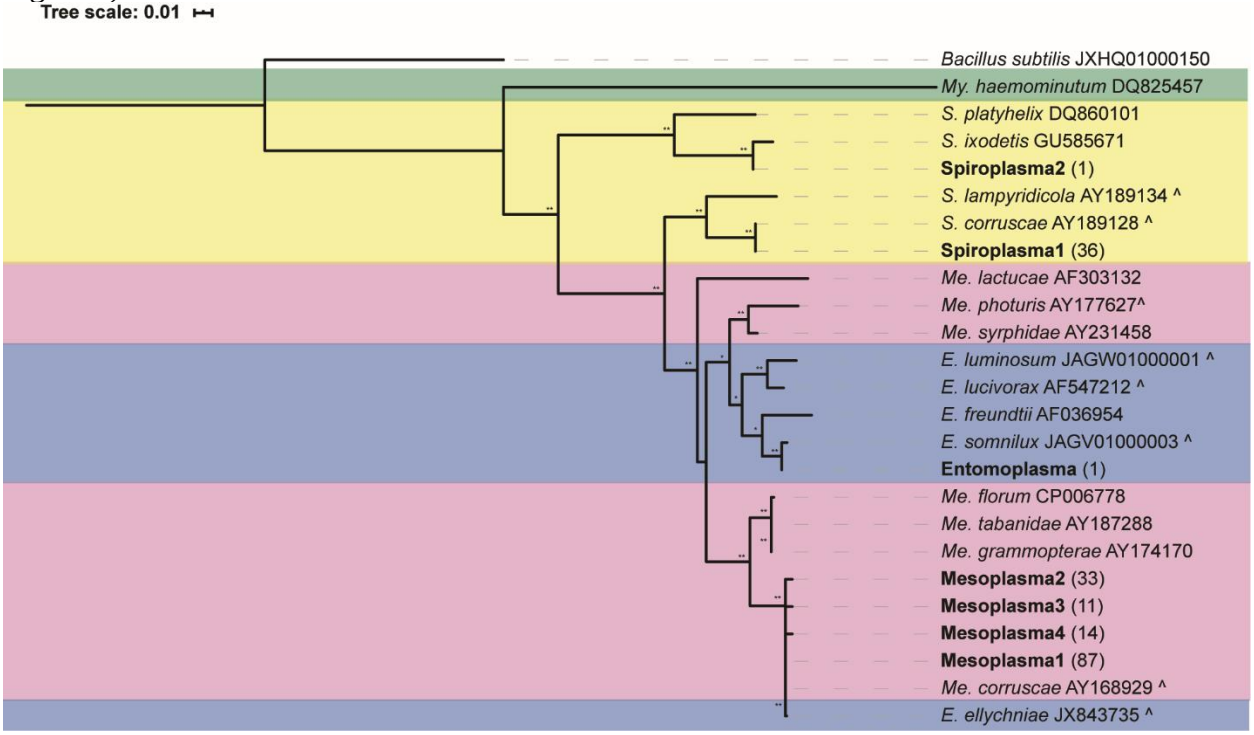


615

Figure 4)



618 Figure 5)



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