Species-specific bacterial associations emerge from stochastically assembled microbiomes in Northeastern American fireflies

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Abstract

Many insects harbor diverse microbial communities ("microbiomes") that can profoundly influence the biology of their host. Yet, for most insect taxa, the relative roles of stochastic events versus deterministic forces in shaping these communities remain unknown. We investigated microbiome assembly in fireflies (*Coleoptera*: *Lampyridae*) using 16S rRNA gene amplicon sequencing and quantitative PCR from 12 species and species groups collected across the Northeastern United States. We hypothesized that firefly microbiomes are predominantly structured by stochastic ecological processes, such as random microbial exposures. Consistent with this, we observed elevated normalized stochasticity ratio (NST) values for most bacteria, coupled with high intraspecific variability in bacterial abundance and composition. However, microbiomes were more similar among closely related firefly hosts, and several bacterial taxa, including endosymbionts and unusually prevalent mollicute strains, showed lower NST values, species-specific associations, and patterns linked to geography, season, and sex. These results reveal that deterministic processes—likely involving host-driven microbial filtering, microbe-microbe interactions, and reliable transmission modes—act alongside stochastic forces to shape firefly-microbe associations. By disentangling these ecological drivers in a diverse, understudied beetle lineage, our study advances understanding of the ecological and evolutionary processes structuring microbiomes in the wild.

Introduction

Eukaryotes are constantly exposed to a plethora of microbes in their environment but only a small fraction of those microbes associate with a host in recurrent symbioses (McFall-Ngai et al. 2013). As in any community of organisms, the assembly of microbial symbiont communities ("microbiomes") associated with eukaryotic hosts is shaped by stochastic (i.e., random) and deterministic processes. Stochastic forces include random spatiotemporal variations, microbial dispersal events, and priority effects (i.e., order of microbial colonizers in hosts) (Jones et al. 2022; Meadow et al. 2013; Sloan et al. 2006; Zhou and Ning 2017). Deterministic forces involve microbial interactions (e.g., competition, facilitation) and host/habitat filtering—defined as the within-host conditions (varying with host species, age, diet, and tissues) or microbial traits (e.g., colonization and persistence abilities) that favor the establishment of certain microbes (Coyte et al. 2021; Mazel et al. 2018; Oliphant et al. 2019). However not all microbial symbionts are the same. Specialized symbionts form species-specific associations (i.e., one microbial strain for one host species), evolving adaptations to a host-associated lifestyle (Kwong and Moran 2015), such as reliable transmission (Michalik and Szklarzewicz 2025). They can influence host physiology and ecology, either in beneficial or harmful ways (Bäumler and Fang 2013; Dillon and Dillon 2003; Hammer 2024), and their establishment is mainly driven by deterministic processes, including host- and/or microbe-derived fidelity-enforcing mechanisms (Bäumler and Fang 2013; Mazel et al. 2018). In contrast, symbiont communities with lower host specialization, typically including microbes acquired from other hosts (i.e., horizontally) or from the environment, provide more flexibility to the host but rely on more hazardous community assembly events, where stochastic processes are predominant (Perreau and Moran 2022). To understand how

62 symbiosis between microbes and eukaryotes emerge and function as stable associations, it is
63 crucial to identify the importance of each of these ecological processes and their interplay.

64 Fireflies (*Coleoptera*: *Lampyridae*) comprise >2,600 species worldwide (Keller 2025), of
65 which 179 have been described in the US and Canada (Fallon et al. 2021; FireflyAtlas.org 2025).
66 Seminal work using culture-based assays has shown that these insects commonly harbor bacteria
67 from the *Mollicutes* class (Hackett et al. 1992; Stevens et al. 1997; Tully et al. 1989, 1994;
68 Williamson et al. 1990), suggesting a role of deterministic processes in developing stable
69 microbe-host associations. This discovery was recently supported through sequencing-based
70 studies which hinted at possible species-specific associations between a few firefly species and
71 certain bacterial genera (Fallon et al. 2018; Green, Smedley, and Klassen 2021). Other microbes
72 have also been sampled from fireflies, including common insect endosymbionts (i.e., symbionts
73 residing inside the body or cells of another organism) (Jeong et al. 2009; Jeyaprakash and Hoy
74 2000) and likely environment-derived bacteria (Green et al. 2021; Zhao et al. 2023), suggesting a
75 role of stochastic processes. However, a more extensive characterization of the firefly
76 microbiome across degrees of relatedness, species lifestyles, diverse environmental factors is
77 required to better comprehend the nature of firefly-bacteria associations and identify the exact
78 contribution of ecological forces to microbiome assembly.

79 While the biology of a large proportion of firefly species is uncharacterized, these insects
80 exhibit diverse life histories and ecologies (e.g., Faust 2017; Lloyd 2018) that can affect, or be
81 affected by, associated microbial communities in deterministic ways. First, as holometabolous
82 insects that include larval, pupal, and adult stages, the ecology and physiology of individual
83 fireflies change drastically throughout their lifetime, potentially resulting in different
84 microbiomes at each developmental stage. In addition, while the adult lifespan is only a few

weeks in most firefly species, it is much longer in some, such as the winter firefly (*Photinus corruscus*) which can overwinter as adults for over 10 months (Deyrup et al. 2017; Rooney and Lewis 2000). Age may be a key factor impacting resistance to bacterial pathogen infections in fireflies (Lower et al. 2023), but little is known about its influence on the firefly microbiome in the wild. Second, while almost all firefly larvae are predators of soft invertebrates like snails (Buschman 1984a, 1984b, 1988; Fu and Benno Meyer-Rochow 2013; Hess 1920; Vaz et al. 2021; Vaz, da Silveira, and Rosa 2020; Yang et al. 2024), adult diets are diverse across species and may impose drastically divergent physico-chemical conditions upon microbial gut colonizers. For instance, members of the *Photuris versicolor* species group prey on other non-photurine fireflies (Lewis, Faust, and De Cock 2011; Lloyd 1965), whereas adult *Pn. corruscus* consume sap and nectar (Rooney and Lewis 2000). Most other firefly species, including *Lucidota* spp., *Pyractomena* spp., and most *Photinus* spp., are assumed not to eat as adults in the wild (Lloyd 2005; Wing 1989; but see Faust 2017; Faust and Faust 2014; Lloyd 1998; Othman et al. 2018). Third, while bioluminescence is used by all firefly species at the larval stage, as a predator avoidance mechanism (Oba et al. 2020; Powell et al. 2022), some species have lost the ability to emit light as adults over evolutionary time (Martin et al. 2017; Stanger-Hall and Lloyd 2015; Stanger-Hall, Lloyd, and Hillis 2007; Zaragoza-Caballero, Zurita-García, and Ramírez-Ponce 2023). Bacterial symbionts are often implicated in bioluminescence of invertebrates (Cassells, Labaude, and Griffin 2024; Jones and Nishiguchi 2004), but nothing is known about microbial involvement in firefly bioluminescence and how microbiomes change between hosts that emit light and those that do not. With all these specificities, fireflies provide a rich system to understand interactions between the microbiota and host ecologies, yet little is known about specialization of microbes to these insects or their functional roles.

108 Here, focusing on field-collected fireflies from 12 common species and species groups
109 from the Northeastern United States (Fig. 1A and 1B), we investigate the degree of host-
110 symbiont specificity and the contribution of diverse factors in structuring firefly microbiomes,
111 such as host species relatedness, diet, bioluminescence, season, geography, urbanization, sex, life
112 stage, and intraindividual tissue localization. Through a combination of quantitative (q)PCR and
113 amplicon sequencing of the bacterial 16S rRNA gene, we tested the hypothesis that most bacteria
114 composing firefly microbiomes are non-specific, and their communities are mainly shaped by
115 stochastic forces, except for host species-restricted bacterial strains from the *Mollicutes* which
116 establish through deterministic processes. We predict that while the relative abundance of most
117 bacteria and total bacterial titers vary in fireflies, each firefly species hosts its own mollicute
118 taxon. This study, focusing on a beetle group that includes differing degrees of symbiont
119 specialization, provides an important contrast to other beetles and paves the way for future
120 research on bacterial transmission modes, functional roles, and influence of microbial symbionts
121 on firefly biology.
122

123 Materials and Methods

124 **1. Specimen collection and identification.** A total of 344 firefly specimens (*Coleoptera*:
125 *Lampyridae*) were collected opportunistically in the continental United States in 2021, 2022, and
126 2023 (Fig. 1A, 1B, and Table S1). We captured specimens by hand or with a net, preserved them
127 in > 95% ethanol upon collection, and froze them within the next 72 hours. Firefly specimens
128 were initially identified and sexed via examination of flash patterns, external morphology, and
129 genitalia (Faust 2017; Fender 1970; Green 1956, 1957; McDermott 1967). To verify species
130 identifications, up to 27 specimens from each species and species group were DNA barcoded, for

131 a total of 59 specimens. We extracted DNA as described below and amplified fragments of the
132 universal metazoan cytochrome C oxidase subunit 1 (COI) gene marker using four different
133 primer pairs (Folmer et al. 1994; Geller et al. 2013; Rennstam Rubbmark et al. 2018; Simon et
134 al. 1994; Stanger-Hall et al. 2007). A full description of the barcoding methods is available in the
135 Supplementary Text 1 with results shown in Fig. S1. DNA barcoding sequences are available as
136 NCBI GenBank files, with accession IDs PV844642-PV844700.

137 The firefly specimens belonged to two subfamilies, four genera, and 12 species or species
138 groups (Fig. 1A). We chose to use species group-level classification for three groups (Photinus
139 consanguineus, Pn. marginellus, and Photuris versicolor) because (*i*) identification to the species
140 level in the field is challenging in some firefly groups based solely on external morphological
141 characters and flash patterns (e.g., *Photuris*; Faust 2017), (*ii*) while male genitalia morphology
142 can be diagnostic in some firefly groups (e.g., *Photinus*; Green 1956), dissection to extract the
143 aedeagus may compromise the tissues needed for microbiome analyses, and (*iii*) species
144 relationships cannot be resolved with the COI gene for some groups (e.g. *Photuris*, some
145 *Photinus*, *Pyractomena*; Lower et al. 2017; Stanger-Hall and Lloyd 2015), either due to lack of
146 variation in the COI locus, incomplete lineage sorting, or lack of COI sequence in GenBank.

147 Among the collected firefly specimens, 332 were adults, including 104 females, 227
148 males, and one *Lucidota atra* specimen that was not sexed, and 12 were juveniles, with nine
149 larvae and three pupae (Fig. 1A). Ten adult beetles from other families were also collected as
150 outgroup specimens. They included three soldier beetles (*Cantharidae*), two tiger beetles
151 (*Cicindelidae*) that were already dead upon collection, three click beetles (*Elateridae*), one net-
152 winged beetle (*Lycidae*), and one fire-colored beetle (*Pyrochroidae*).
153

154 **2. Dissection and DNA extraction.** To study the bacterial communities of fireflies, we extracted
155 DNA from the whole body of 321 specimens. First, fully intact specimens were surface sterilized
156 in 10% bleach, > 95% ethanol, and sterile water. We then flash-froze specimens in empty 1.5 mL
157 tubes by dipping them in liquid nitrogen, before grinding them to powder with a sterile pestle.
158 For each DNA extraction batch (10–23 samples each), a DNA extraction blank sample,
159 consisting of an empty tube, was processed simultaneously with the other samples. DNA was
160 subsequently extracted from flash-frozen, ground samples using the Qiagen DNeasy Blood and
161 Tissue kit (Qiagen, Germantown, MD, USA), following the manufacturer's pretreatment protocol
162 for Gram-positive bacteria.

163 To study the bacterial community of fireflies across tissues, we randomly selected among
164 our preserved specimens 5–6 adult females and males from each of three well-represented firefly
165 taxa (*Photinus corruscus*, *Pn. pyralis*, and the *Pr. versicolor* group; Table S2). We first surface-
166 sterilized the specimens as described above. To isolate tissues, firefly specimens were then
167 dissected in sterile water using bleach-sterilized fine forceps. Separated tissues were placed in
168 pre-chilled 1.5 mL tubes, either filled with 95–100% ethanol or empty, and stored at -80 °C.
169 During the dissections, we separated the entire gut, the reproductive organs, and the lantern (for
170 bioluminescent species) from the rest of the body, which was used as the fourth main tissue (i.e.,
171 "carcass"). To investigate the possibility of endosymbionts (i.e., symbionts residing inside the
172 body or cells of another organism) and/or reproductive manipulators, for a few *Pn. corruscus* and
173 *Pn. pyralis* specimens, we additionally separated some of these main tissues to obtain enlarged
174 fat tissue ("globules"), genitalia, ovaries, hindgut, foregut and midgut, spermatheca,
175 spermatophore-digesting glands, and testes. Dissection blank samples consisted of a firefly leg
176 left in a water droplet on the side of the dissection area. To control for contaminants during

177 dissection, we used a new dissection blank for every one to three dissected specimens. In total,
178 we dissected 33 adult fireflies, resulting in 132 firefly tissue samples and 15 dissection blank
179 samples. Dissection photographs are available in Supplementary Figures. We extracted DNA
180 from dissected tissue samples as described above for whole bodies, but without the surface
181 sterilization step.

182 We obtained a total of 497 DNA samples, including 321 whole-body samples, 132
183 dissected tissue samples, 15 dissection blanks, and 29 DNA extraction blank samples.

184

185 **3. Bacterial titer and total DNA quantifications.** Total bacterial titer per sample was obtained
186 through qPCR (Table S3). CT values were generated with a 7300 Real-Time PCR System
187 (Applied Biosystems, Foster City, CA, USA). We used the 16S rRNA gene as the target,
188 amplified using the bacterial universal 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806R
189 (5'-GGACTACHVGGGTWTCTAAT-3') primers (Apprill et al. 2015; Caporaso et al. 2011;
190 Parada, Needham, and Fuhrman 2016) targeting the V4 region. To obtain absolute bacterial titers
191 measured in 16S rRNA gene copy numbers, we used, in each qPCR plate, a standard curve built
192 on a ten-fold dilution series of a DNA sample with known 16S rRNA gene copy number.
193 Bacterial titers were then corrected using DNA extraction blank and dissection blank samples.
194 Each sample was run in two or three separate technical replicates, consisting of runs on separate
195 qPCR plates. We calculated the average of blank-corrected titers across plate replicates and used
196 these values in downstream statistical analyses. A detailed description of our qPCR procedure
197 can be found in Supplementary Text 1. To normalize bacterial titers and for our Illumina
198 amplicon sequencing decontamination process (see subsection 5.1), we quantified the
199 concentration of extracted DNA per sample using a Qubit 4 fluorometer (Invitrogen, Waltham,

200 MA, USA) and Qubit dsDNA HS and BR kits (Thermo Fisher Scientific, Waltham, MA, USA),
201 with two to three technical replicates per DNA sample.
202
203 **4. Illumina library preparation and sequencing.** A first batch of 20 samples was sequenced at
204 the University of Texas at Arlington Genomic Core Facility in 2021. The library was prepared
205 following Illumina's two-step amplification strategy using primers 341F (5'-
206 CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3'), which
207 amplify the V3-V4 hypervariable region of the 16S rRNA gene (Klindworth et al. 2012), as
208 described in (Ravenscraft et al. 2024). Each sample was bidirectionally sequenced on a 600 cycle
209 paired-end run on an Illumina Mi-Seq platform (Illumina, San Diego, CA, USA). The second
210 batch, containing 477 samples plus one sequencing blank, was sequenced at the Environmental
211 Sample Preparation and Sequencing Facility at Argonne National Laboratory in 2024 (Table S4).
212 The V4 region of the 16S rRNA gene was PCR-amplified with primers 515F and 806R
213 (Caporaso et al. 2012). Amplicons were sequenced on a 300 cycle paired end run in a NextSeq
214 2000 instrument (Illumina) using customized sequencing primers and procedures. For both runs,
215 demultiplexed data were then processed in R (R Core Team 2024) for downstream analysis.
216
217 **5. Bioinformatic pre-treatment.**
218 **5.1. Pre-processing of sequencing data.** For the MiSeq sequencing run, priming sites
219 and poor-quality bases were removed from the 5' and 3' ends of the sequences using the cutadapt
220 v3.2 program (Martin 2011). This step was not necessary for the NextSeq sequences, which did
221 not include priming sites. Then, for each sequencing run, we used the *DADA2* v1.32.0 R package
222 (Callahan et al. 2016) to remove low quality reads, merge paired ends, and infer the bacterial

223 strains present, generating amplicon sequence variants (ASVs). We merged data from the two
224 sequencing runs, performed *de novo* chimera removal, and ran taxonomic classification through
225 the RDP classifier and with a modified SILVA SSU v138.1 training dataset (Quast et al. 2013;
226 Wang et al. 2007). Further details on the pre-processing procedure are available in
227 Supplementary Text 1. Filtered amplicon sequences were exported in fasta format using the
228 *seqinr* v4.2.36 R package (Charif and Lobry 2007) and are available in our data repository
229 (https://doi.org/10.6084/m9.figshare.28306616). Since ASVs from the 20 samples on the MiSeq
230 run were a longer, partially overlapping region of the 16S rRNA and therefore could not be
231 merged with the NextSeq ASVs, we performed all analyses at higher taxonomic levels (e.g.,
232 bacterial genera) and grouped key bacterial taxa phylogenetically (see section 5.2 below).
233 The ASV count table, the taxonomic classification table, and the metadata information
234 (including bacterial titer obtained from qPCR and DNA concentration per sample) were
235 converted into a phyloseq object that contained 99,979 ASVs, using the *phyloseq* v1.48.0 and
236 *speedyseq* v0.5.3.9021 R packages (McLaren 2020; McMurdie and Holmes 2013). Following
237 length filtering (removing 14,757 ASVs and 15% of the sequences), removal of ASVs annotated
238 as chloroplasts, mitochondria, or unidentified at the phylum level (removing 6,778 ASVs and 8%
239 of the remaining sequences), and removal of ASVs only obtained from sequencing blank and
240 extraction blank samples (removing 23,724 ASVs and 31% of the remaining sequences), we
241 used the *decontam* v1.24.0 R package (Davis et al. 2018), with the stringent "either" frequency
242 or prevalence method to discard contaminant ASVs. With this method, 1,555 ASVs were flagged
243 as contaminants and removed (removing 2% of the remaining sequences; list available in our
244 data repository: https://doi.org/10.6084/m9.figshare.28306616), resulting in 53,165 ASVs being
245 retained in our dataset. While we removed a high number of ASVs classified as common

contaminants with this method (e.g., *Klebsiella*, *Pseudomonas*, *Salmonella*, *Staphylococcus*), we note that it removed an abundant ASV from the *Acinetobacter* genus; similar studies have also sequenced *Acinetobacter* from fireflies and it is possible this is a common environmental colonist of firefly guts (Green et al. 2021). In addition, a list of bacteria that passed our decontamination pipeline but that could conceivably be contaminants, because they were only relatively abundant in specimens that had a very low bacterial titer, is shown in Table S5 with more details in Supplementary Text 1.

Finally, to control for among-sample differences in sequencing depth, our data were rarefied to 30,000 sequences per sample using *phyloseq* (Schloss 2023, 2024; Weiss et al. 2017). Visual inspection of rarefaction curves indicated that observed diversity had plateaued in the samples by this depth. Because rarefying would remove important samples in our dissected tissue dataset, we instead used compositional normalization for tissue comparative analyses, computed with the *microViz* v0.12.4 R package (Barnett, Arts, and Penders 2021). Similarly, rarefied data were not used for our multivariate ordination analyses based on Bray-Curtis distances, since compositional normalization has been shown to be a more robust normalization method for clustering on Bray-Curtis distance matrices (McMurdie and Holmes 2014) and because rarefying caused some species to be removed from our dataset (data from our two *Pyractomena* sp. near *sinuata* whole body specimens did not pass the rarefaction threshold).

5.2. Taxonomic annotation refinement through phylogenetic analyses. We next performed phylogenetic analyses to refine the taxonomic identification of abundant and prevalent bacterial clades in fireflies (*Mollicutes*; Fig. S3), common arthropod endosymbionts (*Rickettsiaceae*, *Rickettsiella*, and *Wolbachia*; Fig. S4), and insect pathogens (*Serratia*; Fig. S4). For the *Mollicutes*, we used the terminologies from (Gupta, Son, and Oren 2019), although other

269 authors classify most of the firefly-associated non-*Spiroplasma* mollicutes as the *Entomoplasma*
270 genus (Balish et al. 2019; Gasparich and Kuo 2019; Lo, Gasparich, and Kuo 2018; Yan et al.
271 2024) (but see Arahal et al. 2022:112–122; Gupta et al. 2018; Gupta and Oren 2020). Because
272 our data came from two sequencing batches that used two different amplicon lengths, our
273 phylogenetic analysis allowed us to group together ASVs from the same microorganism that
274 were classified as different variants due to different amplicon sizes. We use the term "bacterial
275 phylotype" hereafter in this section, approximately equivalent to species, to refer to this
276 classification, which we performed only for the five aforementioned bacterial clades (*Mollicutes*,
277 *Rickettsiaceae*, *Rickettsiella*, *Serratia*, and *Wolbachia*).
278 To build these phylogenies, we first selected the 16S rRNA gene amplicon sequence of
279 the most abundant ASVs (those numbered from sv0001 to sv1500, sorted by abundance in our
280 dataset) that were classified as either from the *Mollicutes* class (i.e., *Entomoplasmatales* and
281 *Mycoplasmatales* orders *sensu* SILVA database), from the non-*Anaplasmataceae Rickettsiales*
282 order (i.e., including the *Rickettsiaceae* family, unidentified *Rickettsiales*, and *Candidatus*
283 Hepatincola), or from one of the focal bacterial genera (*Rickettsiella*, *Serratia*, and *Wolbachia*).
284 We used these sequences in NCBI BLASTn runs and downloaded the 16S rRNA gene sequence
285 of the closest matches. We also downloaded the sequences of reference species from NCBI. For
286 the *Mollicutes*, representative amplicon sequences of each of the firefly-associated bacterial
287 species found in Green et al. (2021) were also used, as well as a few insect-associated mollicute
288 sequences from Funaro et al. (2011). With all these sequences, we produced Clustal Omega
289 (Sievers et al. 2011) multiple sequence alignments in SeaView v5.0.5 (Gouy, Guindon, and
290 Gascuel 2009), which were used to build maximum likelihood phylogenies using RAxML
291 Blackbox (Stamatakis 2014), implemented in the CIPRES Science Gateway (Miller, Pfeiffer, and

292 Schwartz 2010), with 999 bootstrap iterations. All phylogenetic trees were visualized and
293 manually annotated in iTOL (Letunic and Bork 2016). Further details on these phylogenetic
294 methods can be found in Supplementary Text 1.

295 Finally, we manually edited the *phyloseq* taxonomy table to account for this taxonomic
296 refinement (available as a Data Repository: https://doi.org/10.6084/m9.figshare.28306616).
297 Except for analyses on alpha diversity metrics and specific mentions of analyses run at the
298 bacterial order or genus levels, all downstream analyses were run on microbiome data aggregated
299 at the genus level, except for the aforementioned groups we refined (*Mollicutes*, *Rickettsiaceae*,
300 *Rickettsiella*, *Serratia*, and *Wolbachia*) which were aggregated at the "phylotype" level (Fig. S2).
301 This combination of genus and phylotype level classification is hereafter referred to as "taxon"
302 level. Data was aggregated at the bacterial order, genus, and taxon levels using the *microViz* R
303 package.

304

305 **6. Bioinformatic analyses.**

306 **6.1. Stochasticity analyses**. To infer the relative importance of stochastic *vs.*
307 deterministic processes contributing to microbiome assembly, we calculated the normalized
308 stochasticity ratio (i.e., NST index) for each firefly species that had more than two specimens in
309 our dataset through implementation of null-models using the *NST* R package v3.1.9 (Ning et al.
310 2019). An index value between 0.5 and 1 suggests that stochastic processes shape community
311 assembly, whereas a value between 0 and 0.5 indicates higher influence of deterministic
312 processes. Next, to infer the contribution of specific bacterial groups, we tested how the removal
313 of either all the mollicutes, all putative endosymbionts (*Ca.* Hepatincola, *Rickettsia*, *Rickettsiella*,
314 and *Wolbachia*), or both, from the microbiome data of each firefly species affects the NST index

315 value. We generated 95% confidence intervals via bootstrapping and tested NST variation
316 between the unaltered microbiome of firefly species *vs.* that following removal of the
317 aforementioned bacterial groups through pairwise, two-tailed Wilcoxon signed-rank statistical
318 tests (Table S6).

319 **6.2. Alpha and beta diversity**. To assess patterns of diversity, richness, evenness,
320 dominance, and rarity in bacterial communities from fireflies, indices of alpha diversity were
321 calculated on rarefied data with non-aggregated (ASV level) taxonomic tables, using the
322 *microbiome* v1.26.0 R package (Lahti and Shetty 2017) (Table S7). To compare bacterial
323 composition across firefly species, genera, subfamilies, and diverse factors, we used beta
324 diversity metrics, first running ordinations on Bray-Curtis distance matrices computed from non-
325 rarefied, proportional abundance data using nonmetric multidimensional scaling (NMDS)
326 analyses computed with *phyloseq*, to infer similarities among fireflies from different taxa, diets,
327 adult use of bioluminescence, regions, and tissues. To test how different factors were correlated
328 with bacterial composition across fireflies, we then ran permutational multivariate analysis of
329 variance (PERMANOVAs) on rarefied, taxon-aggregated data, with the *vegan* v2.6.4 R package
330 (Oksanen et al. 2016), and pairwise PERMANOVAs as post hoc tests using the *pairwiseAdonis*
331 v0.4.1 R package (Martinez 2020).

332 **6.3. Host relatedness, geographic distance, and microbiome dissimilarity**. The impact
333 of firefly host phylogeny on bacterial community dissimilarity was assessed using Mantel tests.
334 We first generated a host phylogeny using five previously sequenced firefly genes (Catalan et al.
335 2022; Martin et al. 2017; Sander and Hall 2015; Stanger-Hall and Lloyd 2015; Stanger-Hall et al.
336 2007) (Supplementary Text 1 and Fig. S1). We then used the *ape* R package (Paradis, Claude,
337 and Strimmer 2004) to generate a phylogenetic distance matrix from our tree with the

338 cophenetic.phylo() function. We next produced a matrix of geographic distance among collected
339 firefly specimens calculated from latitude and longitude decimal variables at collection using the
340 *sf* v1.0.17 R package (Pebesma 2018). As proxies for microbiome dissimilarity, we generated
341 two pairwise distance matrices using the Bray-Curtis distance from rarefied relative bacterial
342 abundance between firefly specimens and the Bray-Curtis binary distance from bacterial
343 presence/absence (equivalent of Sørensen-Dice distance) between firefly specimens. Bacterial
344 presence was defined as any bacterial taxon with at least 1% relative abundance in a specimen
345 and absence as less than 1% relative abundance in that specimen. Because the number of
346 pairwise comparisons was not computationally tractable, we applied a filter to our dataset,
347 keeping only bacterial taxa that had more than 0.5% relative abundance in at least 5% of all
348 firefly specimens. These microbiome dissimilarity matrices were compared to a matrix of
349 phylogenetic distance and to geographic distances between firefly specimens through Mantel and
350 partial Mantel tests using the *vegan* R package, allowing us to control for host phylogeny and
351 geography, respectively.

352 **6.4. Contribution of firefly ecological traits to bacterial abundance and diversity**. We
353 tested the influence of adult bioluminescence, diet, and body size of firefly species on bacterial
354 abundance and diversity, as well as correlations between bacterial abundance and diversity
355 metrics via generalized least square analyses with the *nlme* v3.1.164 R package (Pinheiro, Bates,
356 and R Core Team 2024). To account for the effects of phylogenetic nonindependence in
357 comparisons of firefly species-level covariates, we used phylogenetic generalized least square
358 (PGLS) analyses, fitting a Brownian motion model of evolution. The firefly phylogeny was
359 obtained as described in section 6.3 and Supplementary Text 1 (Fig. S1). Bacterial titer and
360 diversity indices were averaged per firefly species. We also averaged body size (i.e., total adult

361 male body length) per firefly species or species groups using the ranges from Faust (2017),
362 Fender (1970), Green (1956, 1957), and Lloyd (1969).

363 **6.5. Bacterial composition and differential abundance analysis**. To test whether single
364 bacteria vary in their abundance across fireflies and factors, we used the *MaAsLin2* v1.15.1 R
365 package (Mallick et al. 2021), fitting our rarefied (or non-rarefied for tissue analyses) data to
366 negative binomial distributions and controlling for random effects (see section 6.7. Statistical
367 tests) in multiple generalized linear mixed models. To account for variables with unbalanced
368 discrete levels (e.g., species with high *vs.* low number of specimens sampled), when more than
369 two discrete levels were present in our fixed effect(s), we ran the same *MaAsLin2* models
370 multiple times, changing the reference level each time (Tables S8–S11). We filtered out bacterial
371 taxa detected in less than 1% of all firefly samples being tested, unless the test was run on groups
372 with very low sample size (prevalence threshold lowered to 0.1% instead).

373 **6.6. Bacterial co-occurrence analysis**. To investigate positive and negative co-
374 occurrence patterns among bacteria in adult fireflies, we ran co-occurrence analyses, within
375 firefly species that had more than eight adult individuals in our dataset (i.e., excluding *Lu. atra*,
376 *Pn. consanguineus* group, *Pn. cookii*, and *Py. borealis*) and grouping all *Photuris* together. We
377 used both the *cooccur* v1.3 and *CooccurrenceAffinity* v1.0 R packages (Griffith, Veech, and
378 Marsh 2016; Mainali and Slud 2022). A filter was applied before analyses to obtain a
379 manageable number of bacterial taxa to be tested per firefly species. Bacterial taxa were
380 considered present when they had at least 1% relative abundance (absent otherwise) in a
381 specimen and were kept in these analyses when they had more than 10,000 reads—or 20,000
382 reads for the *Photuris*—in one or more samples for a given firefly species or species group.

383 **6.7. Statistical tests**. In addition to the tests described in the previous sections,
384 differences in alpha diversity and qPCR titers across hosts and diverse environmental factors
385 were assessed using linear mixed models (LMM) with the *lme4* v1.1.35.5 R package (Bates et al.
386 2015), which allowed us to control for random effects that may otherwise bias our interpretation
387 of statistical tests. Some key factors were always kept as random effects in our models because
388 they had a strong influence on the microbiome of fireflies in our datasets. These random factors
389 included the year of collection, the geographic location of sampling (either address, longitude,
390 latitude, or region), and collection day or month of year (a rough proxy for firefly age in many
391 species). When they were not among the fixed effects in our models, host sex (which included
392 developmental stages) and species were used as random effects or covariates. The importance of
393 other random effects in our models was previously tested through bidirectional model selection
394 with likelihood ratio tests and comparing model's AICs.
395 We tested the distribution of the residuals, percentage of the dispersion of the data, the
396 presence of outliers, and zero-inflation using the *performance* v0.12.3 and *DHARMa* v0.4.6 R
397 packages (Hartig 2018; Lüdecke et al. 2021). Our models were often flagged as poor fits due to
398 zero-inflation, despite normalization and log10 transformation. When this was the case, we fitted
399 our data to a negative binomial distribution in generalized LMMs (GLMMs) using *lme4*. We
400 next performed pairwise post hoc tests using the *emmeans* v1.10.4 R package (Lenth 2022) to
401 compute the estimated marginal means (Searle, Speed, and Milliken 1980). In doing so, we
402 applied an asymptotic degrees-of-freedom method, thus invoking *z*-statistics, which are
403 computationally faster and recommended with larger sample sizes. A description of all LMMs,
404 GLMMs, PERMANOVAs, pairwise PERMANOVAs, Mantel tests, OLS, and PGLS models is
405 available in Table S12.

406

407 Additional files, including sequencing OTU and taxonomy tables, list of contaminant
408 variants removed, multiple sequence alignments, photographs of insect specimens, multi-locus
409 backbone phylogenetic trees, R script and input files, and additional supplementary figures, are
410 available in our data repository at https://doi.org/10.6084/m9.figshare.28306616.

411

412 ## Results

413 **1. The microbiome of fireflies is mostly structured by stochastic processes, except when**
414 **bacteria from the *Mollicutes* are included.** To assess the relative importance of deterministic
415 *vs.* stochastic factors in structuring firefly bacterial communities, we calculated and compared
416 the normalized stochastic ratio (NST; Ning et al. 2019) across species that had more than two
417 specimens in our dataset. We found that the microbiome of all nine firefly species tested had an
418 NST index below 0.5 (average = 0.27, SD = 0.10, median = 0.26; Fig. 1C and Table S6),
419 indicating that their microbiome is structured by deterministic processes. To infer which
420 bacterial group may be responsible for deterministic community assembly, we next removed all
421 the *Mollicutes* from our datasets, and found that the NST significantly increased for five out of
422 nine firefly species (Wilcoxon signed-rank test for these five species: $W < 2.3 \times 10^4$, $P < 0.05$),
423 reaching index values above 0.5 (for these five species: average = 0.62, SD = 0.09, median =
424 0.58). This suggests that for several firefly species, while stochastic factors are involved in
425 structuring most of the bacterial community, deterministic processes shape the relative
426 abundance of mollicutes, influencing the whole microbiome. Interestingly, this was not the case
427 for members of the *Photinus marginellus* group, for which removing bacteria known as
428 endosymbionts (i.e., symbionts residing inside the body or cells of another organism) of

429 arthropods (i.e., *Candidatus* Hepatincola, *Rickettsia*, *Rickettsiella*, *Wolbachia*) instead
430 significantly increased the NST index, reaching a value of 0.56 (Wilcoxon signed-rank test: $W =$
431 370, $P < 0.001$). This indicates that for this firefly species group, deterministic processes shape
432 the abundance of endosymbionts other than mollicutes.
433
434 **2. The microbiome of fireflies is phylogenetically structured.**
435 **2.a. Firefly genera differ in their bacterial density and diversity.** Using qPCR, we
436 found that *Photuris* fireflies had significantly higher bacterial densities than *Photinus* fireflies
437 (emmeans: z-ratio = 4.64, $P < 0.0001$), despite high intraspecific variability (Fig. 1D; Fig. S5).
438 The *Pyractomena* and *Lucidota* genera were much less represented in our dataset and members
439 of these groups appear to have intermediate but variable bacterial levels. When looking within
440 firefly genera, we found that *Pn. corruscus* differed from other *Photinus* species in that
441 specimens from this long-lived adult, day-active species had three orders of magnitude higher
442 bacterial titers, reaching levels close to those of *Pr. lucicrescens* (Fig. 1D and S5). The
443 difference between *Pn. corruscus* and other *Photinus* species was, however, only significant
444 when comparing bacterial titers of *Pn. corruscus* to *Pn. pyralis* (emmeans: z-ratio = 3.67, $P <$
445 0.05). This indicates that, with the exception of *Pn. corruscus*, members of the *Photinus* genus
446 tend to be infected with low to extremely low bacterial densities in the wild, while *Photuris*
447 fireflies have moderately high to very high bacterial titers.
448 In terms of bacterial richness, despite having relatively low bacterial densities, fireflies
449 from the *Photinus* genus harbored significantly more bacterial ASVs than *Photuris* fireflies
450 (emmeans: z-ratio = 4.38, $P < 0.001$; Fig. 1E and S6, Table S7), suggesting that fireflies with
451 higher bacterial densities tend to harbor fewer bacterial species.

452 **2.b. Bacterial composition differs across firefly subfamilies, genera, and species.**
453 Following rarefying and aggregation at the bacterial taxon level, we tested differences across
454 microbiome compositions. The microbiome of Northeastern fireflies differed between the
455 *Lampyrinae* and *Photurinae* subfamilies (PERMANOVA: $R^2 = 0.07$, $F_{1,264} = 22.95$, $P < 0.001$;
456 Fig. 2A) and across genera (nested within subfamilies, PERMANOVA: $R^2 = 0.02$, $F_{2,264} = 3.87$,
457 $P < 0.001$; Fig. 2B). Despite high intraspecific variability, bacterial composition also differed
458 significantly across firefly species (nested within genera, within subfamilies, PERMANOVA: R^2
459 $= 0.09$, $F_{7,264} = 4.18$, $P < 0.001$; Fig. 2C–F). To assess whether firefly microbiomes were more
460 divergent in species that were less genetically related, we examined how bacterial community
461 composition varied with degree of host relatedness. We found a strong positive correlation
462 between pairwise microbiome dissimilarities and host genetic divergence, with closely related
463 firefly species having more similar microbiome composition and abundance (partial Mantel test
464 controlling for geographic distance: $r = 0.37$, $P < 0.001$; Fig. S7), further indicating
465 phylogenetically structured microbiomes in fireflies.
466 **2.c. Firefly species that include individuals with bigger size tend to harbor higher**
467 **bacterial densities.** We investigated whether three key characteristics of firefly species—the use
468 of bioluminescence as a mating signal by adults, adult diet, and body size—influenced firefly
469 microbiomes when taking into account fireflies' degree of relatedness. Through phylogenetic
470 generalized least square models, we found that bacterial density, richness, and composition did
471 not significantly change between bioluminescent and non-bioluminescent fireflies and between
472 species eating and not eating at the adult stage (Fig. S7 and S8; Supplementary Text 1). We also
473 found that bigger firefly species tend to harbor a higher bacterial density and a lower bacterial
474 richness than smaller species (Fig. S9; Supplementary Text 1). These results show that firefly

475 microbiomes do not appear to be influenced by mating signals, adult diet, but that species' body
476 size may contribute.
477
478 **3. Firefly microbiomes are often dominated by a few bacterial taxa with signs of species-**
479 **specific associations.** The most abundant bacterial order associated with all fireflies in our
480 dataset was *Entomoplasmatales* (class *Mollicutes*), representing 27.3% of all rarefied bacterial
481 reads from fireflies. Highly represented bacterial orders in fireflies also included *Burkholderiales*
482 (9.5% of all rarefied reads), *Xanthomonadales* (8.6%), *Pseudomonadales* (8.3%),
483 *Enterobacterales* (7.7%), and *Rickettsiales* (7.0%). Compared to non-lampyrid elateroid beetles,
484 only a few of the abundant firefly-associated bacterial taxa were similar (Fig. S10;
485 Supplementary Text 1).
486 **3.a. Fireflies harbor a diversity of mollicute taxa with prevalent and abundant**
487 **strains and a few endosymbionts.** The fireflies we collected harbored strains from almost all
488 the mollicute taxa that have been sampled from fireflies in the past (7 out of 8; Fig. 3 and S3;
489 Supplementary Text 1). Both *Spiroplasma ixodetis* and *Williamsoniiplasma lucivorax* species
490 were represented with a high strain diversity in our firefly specimens, with at least 20 and 30
491 ASVs each, respectively. Within several mollicute genera and species, one or two ASVs could
492 be very abundant and prevalent in fireflies, some of which have only been sampled from firefly
493 bodies to date and thus appear endemic to fireflies. While the high abundance of mollicutes
494 associated with fireflies was unusual for an insect microbiome, we note that several prevalent
495 and abundant bacteria in these insects are known to be endosymbionts in other arthropods (Fig.
496 4A). These include bacteria from the *Rickettsia*, *Rickettsiella*, *Wolbachia*, and occasionally
497 *Serratia* and *Spiroplasma* genera (Russell et al. 2013; Werren, Baldo, and Clark 2008).

498 **3.b. *Stenotrophomonas* and *Williamsoniiplasma* often dominate lampyrine**
499 **microbiomes.** Following filtering of bacterial taxa with low abundance (keeping only taxa with
500 rarefied abundance > 20,000 reads in one or more samples), we found that the *Stenotrophomonas*
501 (*Xanthomonadales*) and *Williamsoniiplasma* (*Mollicutes*) genera were characteristic members of
502 the microbiome of fireflies from the *Lampyrinae* subfamily (Fig. 4B), each detected in more than
503 98% of lampyrine fireflies (colonizing > 57% of lampyrine specimens with more than 1%
504 relative abundance). Compared to fireflies from the *Photurinae* subfamily, lampyrine fireflies
505 were significantly more associated with bacteria from these two genera (MaAsLin2: coef > 3.30,
506 FDR *P*-adj < 0.0001). While *Stenotrophomonas* could also be found in *Photuris* fireflies, albeit
507 typically at low abundance, members of the *Williamsoniiplasma* genus were very rare in
508 *Photuris* species (Fig. 4A, 4B, S11, and S12), making this bacterial genus nearly endemic to the
509 lampyrine microbiome.
510 Three *Williamsoniiplasma* taxa were commonly detected in *Photinus* and *Pyractomena*
511 fireflies with differing levels of endemicity (Fig. 4A and S11). *Wi. lucivorax* was only found in
512 *Pn. pyralis*, as the most dominant and prevalent bacterium after *Stenotrophomonas*, infecting
513 over 69% of individuals with more than 1% relative abundance, and being significantly more
514 abundant in this firefly species than in any other (MaAsLin2: coef > 4.50, FDR *P*-adj < 0.0001;
515 Fig. S11 and Table S8). In contrast, *Wi. luminosum* was detected in both *Pn. pyralis* and *Pn.*
516 *scintillans*, as well as in two individuals from the *Pn. marginellus* group (Fig. 4A and S11), thus
517 appearing less host-restricted than *Wi. lucivorax*. Yet, *Wi. luminosum* was significantly
518 associated with *Pn. scintillans* (*vs.* all firefly species but *Pn. marginellus* group: MaAsLin2: coef
519 > 1.55, FDR *P*-adj < 0.05; Fig. S11), being found in all *Pn. scintillans* specimens and 64% of *Pn.*
520 *scintillans* specimens with 1% relative abundance threshold (Fig. 4A). Another species from the

521 *Williamsoniiplasma* genus, *Wi. somnilux*, was significantly more abundant in *Pyractomena*
522 *borealis* than in other firefly genera (MaAsLin2: coef > 8.04, FDR *P*-adj < 0.0001; Table S8)
523 and showed endemicity patterns similar to those of *Wi. lucivorax* (Fig. 4A and S11). In our eight
524 specimens from this firefly species, including adults and juveniles, *Wi. somnilux* was present in
525 five out of eight specimens (62%) when using the 1% relative abundance threshold and was the
526 most abundant bacterial taxon (Fig. 4A). We also found that *Pn. corruscus* fireflies were
527 significantly associated with another member of the *Mollicutes*, the *En. corruscae* taxon
528 (MaAsLin2: coef > 4.30, P-adj < 0.001; Fig. 4A and S11), being the most abundant bacterial
529 taxon in all *Pn. corruscus* fireflies, found at more than 1% relative abundance in nine out of 16
530 individuals (56%), and showing patterns of endemicity as well.

531 In contrast, four other lampyrine species and species groups rarely harbored mollicutes.
532 Instead, in the *Pn. marginellus* group, *Wolbachia* (*Rickettsiales*) from the supergroup A was
533 found to be a dominant bacterium (colonizing > 47% of specimens with more than 1% relative
534 abundance).

535 **3.c. Photurine fireflies commonly harbor a variety of bacteria, including a few**
536 **mollicutes.** Contrasting with the lampyrines, members of the *Photurinae* were significantly more
537 associated with abundant bacteria (filtered taxa with > 20,000 reads in one or more samples)
538 from the *Achromobacter* (*Burkholderiales*), *Ca.* Hepatincola (*Rickettsiales*), *Curvibacter*
539 (*Burkholderiales*), *Elizabethkingia* (*Flavobacteriales*), *Enterococcus* (*Lactobacillales*), and
540 *Spiroplasma* (*Mollicutes*) genera (MaAsLin2: coef > 1.87, FDR *P*-adj < 0.0001; Fig. 4B).
541 Despite a lower relative abundance, *Leucobacter* bacteria also appeared significantly associated
542 with photurine fireflies (MaAsLin2: coef = 2.97, FDR *P*-adj < 0.0001; Fig. S11, S12, and Table
543 S8). Among the spiroplasmas, two taxa were significantly more abundant in photurine fireflies:

544 *Sp. ixodetis* and *Sp. lampyridicola* (found in 14% and 7% of *Photuris* specimens at 1% relative
545 abundance threshold, respectively) (MaAsLin2: coef > 3.25, FDR *P*-adj < 0.0001). Another
546 mollicute species, *Tullyiplasma photuris*, was abundant in photurine fireflies, though at lower
547 degrees (MaAsLin2: coef = 0.69, FDR *P*-adj < 0.05; Fig. 4A and S11), found in 13% of *Photuris*
548 specimens at 1% relative abundance threshold.

549 **3.d. Bacteria positively and negatively co-occur in fireflies.** From our co-occurrence
550 analysis, we found that some bacteria, including endosymbionts (e.g., *Rickettsia bellii*,
551 *Wolbachia*) and potential pathogens (e.g., *Serratia marcescens-nematodiphila*), were more likely
552 to be found on their own in fireflies (Fig. S13). We also found some positive co-occurrence
553 patterns within firefly species, including *Pseudomonas*, *Stenotrophomonas*, and *Rl. costelytrae* in
554 both *Pn. marginellus* group and *Pn. pyralis*, and *Achromobacter*, *Curvibacter*, and
555 *Elizabethkingia* in *Photuris* fireflies. This suggests that some bacteria are more likely to occur
556 alone whereas others may be part of a consortium in fireflies.

557

558 **4. Within-species variation can be partially explained by the region, season, environment,**
559 **sex, and developmental stage.**

560 **4.a. The abundance of some, but not all, bacteria vary across geographic locations.**
561 To assess whether geography was a main factor influencing changes in firefly microbiomes, we
562 first used partial Mantel tests on microbiome dissimilarity matrices and geographic distance,
563 while taking into account species' phylogenetic distances. Through this test, we found a weak,
564 positive correlation between pairwise microbiome dissimilarities based on relative bacterial
565 abundance and geographic distance (partial Mantel test controlling for genetic distances: r =
566 0.05, $P < 0.05$), indicating that regardless of host species identity, fireflies separated by longer

567 spatial distances tend to harbor more dissimilar microbial communities. Because of substantial
568 intra-specific variation in bacterial density and community composition, we next investigated
569 within species microbiome variation for species that were collected in multiple regions.

570 At the level of the whole bacterial community, *Pn. corruscus* microbiomes did not appear
571 to differ across regions (PERMANOVA: $R^2 = 0.40$, $F_{5,10} = 1.33$, $P = 0.11$; Fig S14).
572 Nevertheless, and despite a low sample size ($n = 16$) and potential confounding factors (albeit
573 being controlled as random factors in our GLMMs and MaAsLin2 models), we found regional
574 differences in terms of absolute bacterial abundance (Supplementary Text 1) and at the level of
575 individual bacteria (Fig. 5A and Table S9). *En. corruscae*, which was a characteristic bacterial
576 taxon of *Pn. corruscus*, may have preferentially been associated with specimens from northern
577 latitudes, since it was less abundant in specimens collected in southwestern Connecticut (swCT)
578 and South New Jersey compared to specimens collected in northwestern CT (nwCT),
579 southeastern New York (seNY), and southwestern Vermont (swVT) (MaAsLin2: coef > 4.57,
580 FDR P-adj < 0.01).

581 *Pn. pyralis* microbiomes showed significant variation in composition across regions
582 (PERMANOVA: $R^2 = 0.13$, $F_{5,106} = 3.15$, $P < 0.001$; Fig. S14), despite relatively stable absolute
583 bacterial abundances (emmeans: z-ratio < 1.76, $P > 0.07$). The most divergent microbiomes were
584 from *Pn. pyralis* specimens collected in central Pennsylvania (cPA) (pairwise adonis: $R^2 > 0.08$,
585 $F > 2.99$, FDR P-adj. < 0.05; Fig. 5B). Likely contributing to the singularity of cPA *Pn. pyralis*
586 microbiomes, *Wi. luminosum* was mostly found in individuals collected in this region
587 (MaAsLin2: coef > 3.64, FDR P-adj < 0.05), where its prevalence at 1% relative abundance
588 threshold was 68% (64% for *Wi. lucivorax* in these specimens) *vs.* 4% in *Pn. pyralis* from all
589 other regions (71% for *Wi. lucivorax* in these other specimens). For *Wi. lucivorax*, relative

590 abundance was high in specimens collected in several states. *Pn. pyralis* specimens from western
591 Virginia also had a divergent microbiome compared to specimens from other regions, with
592 compositions differing from those of cPA and southeastern PA specimens (pairwise adonis: R^2 =
593 0.03, F = 2.51, FDR P-adj. < 0.05), and unusually dominated by bacteria from the *Mycoplasma*-
594 related group (Fig. 5B). For both *Pn. marginellus* group and *Pn. scintillans*, we found limited
595 evidence of regional specificities in the microbiome (PERMANOVA: R^2 < 0.17, F < 1.67, P >
596 0.11; Fig 5B and S14), although total bacterial density was higher in *Pn. scintillans* collected in
597 cPA compared to sePA (emmeans: z-ratio = 2.03, P < 0.05).

598 Regarding the *Photuris* genus, we found strong microbiome differences across regions
599 for the unidentified species of the *Pr. versicolor* group (PERMANOVA: R^2 = 0.13, $F_{4,51}$ = 1.83,
600 P < 0.001; Fig. S14), but not for *Pr. hebes* (PERMANOVA: R^2 = 0.11, $F_{2,19}$ = 1.18, P = 0.24).
601 *Sp. ixodetis* bacteria were common and abundant in specimens collected in cPA, whereas these
602 bacteria were very rare in specimens from other regions (MaAsLin2: coef > 5.58, FDR P-adj <
603 0.001). We also identified regional specificities among other common bacterial associates of
604 *Photuris* fireflies, with *T. photuris* being more abundant in northeastern NY (neNY) and seNY
605 compared to sePA for *Pr. hebes* (MaAsLin2: coef > 5.88, FDR P-adj < 0.0001) and neNY
606 compared to sePA in unidentified species from the *Pr. versicolor* group (MaAsLin2: coef > 3.53,
607 FDR P-adj < 0.05).

608 **4.b. Seasonal, but not annual, factors influence within-species microbiome variation.**
609 We tested whether seasonality (which is linked to firefly adult age) influenced firefly
610 microbiome. When focusing on all species in our dataset, we found that bacterial density varied
611 throughout day of year only in some firefly species (GLMM Wald test: X^2 = 124.38, P < 0.0001).
612 Several firefly species, including *Pn. scintillans*, unidentified *Pr. versicolor* group, and *Py.*

613 *borealis*, tended to have higher bacterial titers later in the season (emmeans: z-ratio > 2.63, $P <$
614 0.05). We also found that composition varied throughout the year and across species
615 (PERMANOVA with species and regions as covariates, focusing on the interaction with day of
616 year: species: $R^2 = 0.05$, $F_{14,186} = 1.20$, $P < 0.05$). We next focused our analyses on only *Pn.*
617 *corruscus* specimens given their long adult lifespan (up to 10 months *vs.* less than three months
618 usually for other species) and since they were collected throughout the year.

619 While the overall composition of *Pn. corruscus* microbiomes changed marginally across
620 months of collection (PERMANOVA: $R^2 = 0.27$, $F_{3,12} = 1.52$, $P = 0.070$), the specimens
621 collected in April, May, and June had higher bacterial titers than specimens collected in
622 November (emmeans: z-ratio > 3.55, $P < 0.01$; Fig. 6A). When looking at individual bacterial
623 taxa, we found that specimens collected in May and April were typically associated with high to
624 very high relative abundance of *En. corruscae*, but this bacterial taxon was barely detectable in
625 *Pn. corruscus* specimens collected in February and November (April and May *vs.* February and
626 November, MaAsLin2: coef > 4.89, FDR P-adj < 0.01; Fig. 6B). Supporting these results across
627 months, we found a strong association between the collection day of year used as a quadratic
628 continuous predictor in our LMMs and both total bacterial titers (GLMM Wald tests: $X^2 > 35.81$,
629 $P < 0.0001$) and abundance of *En. corruscae* (LMM Wald tests: $X^2 > 18.47$, $P < 0.0001$) in *Pn.*
630 *corruscus*, with a peak in late May (Fig. 6A and 6B). Thanks to citizen science data collected
631 from iNaturalist (iNaturalist community 2025), we found that during the years and in the states
632 of collection, the reproductive activity of *Pn. corruscus* spanned from early April to mid/late
633 June, peaking during the month of May (Fig. 6C). This corresponds to the reproductive seasons
634 observed for this species in other states and years (Faust 2012; Rooney and Lewis 2000;

635 Smedley et al. 2017), and implies a potential link between *En. corruscae* abundance and either
636 its host reproduction or seasonal increased food intake (Rooney and Lewis 2000).

637 To assess microbiome turnover across years, we collected *Pn. pyralis* specimens in the
638 same location (Philadelphia Fairmont Park), at approximately the same time of year, for three
639 consecutive years from 2021 to 2023. We found that the overall microbiome composition of *Pn.*
640 *pyralis* changed only marginally over the years (PERMANOVA: $R^2 = 0.06$, $F_{2,53} = 1.76$, $P =$
641 0.051). However, at the level of individual bacterial taxa, while the abundance of *Wi. lucivorax*
642 remained stable from 2021 to 2023, that of *T. photuris* and *Wi. luminosum* increased and that of
643 *Pseudoclavibacter* and *Tumebacillus* dropped (MaAsLin2: coef < -1.96, FDR *P*-adj < 0.05; Fig.
644 S15). This shows that the overall composition of bacteria associated with fireflies may not
645 drastically change over the years, although the abundance of a few bacteria may fluctuate.

646 **4.c. The microbiome of *Photuris versicolor* group fireflies change across habitat**
647 **types.** For both bacterial density and composition, the interaction between species and habitat
648 types (with different urbanization levels) was significant (PERMANOVA with species and
649 regions as covariates: $R^2 = 0.05$, $F_{14,235} = 1.30$, $P < 0.001$; GLMM Wald test: $X^2 = 143.6$, $P <$
650 0.0001), indicating that different species' microbiomes vary in their response to divergent habitat
651 types. In unidentified species from the *Pr. versicolor* group, we found that absolute bacterial
652 abundance was significantly lower for specimens collected in the city than in the forest or in a
653 mix of forest and meadow (emmeans: *z*-ratio > 3.17, $P < 0.05$; Fig. 6D). Despite the
654 aforementioned confounding factors between regions and habitat types, we found that, when
655 controlling for these factors in our multiple GLMMs, *Rickettsiella costelytrae* may have been a
656 key influence on bacterial titers over the urbanization gradient since it was more abundant in
657 specimens from the forest than in the city (MaAsLin2: coef = 5.72, FDR *P*-adj < 0.01; Fig. S16).

658 These results show that the type of habitat used by *Photuris* fireflies may influence the
659 abundance and composition of their associated bacteria.

660 **4.d. *Photinus* females harbor a denser bacterial community than males.** Using qPCR,
661 we found that females of both *Pn. pyralis* and *Pn. scintillans* had a significantly higher bacterial
662 titer than their male conspecifics (emmeans: z-ratio > 2.03, $P < 0.05$; Fig. 6E). In general, the
663 Evar index of evenness was higher for males compared to females in *Photinus* species
664 (emmeans: z-ratio = 3.75, $P < 0.01$; Fig. S17), suggesting that bacterial strains in male
665 microbiomes were represented in more equal proportions, while female microbiomes tended to
666 be dominated by fewer bacteria. Nevertheless, microbiome composition did not significantly
667 differ between sexes, with the only exception of *Pr. hebes* (pairwise adonis: $R^2 = 0.09$, $F = 1.97$,
668 FDR P-adj < 0.05).

669 **4.e. Juveniles harbor a more diverse bacterial community than adults.** We collected
670 a few larvae from three different firefly genera, and pupae from the *Pyractomena* genus. When
671 comparing adults to juveniles (i.e., including both larvae and pupae) from all firefly species,
672 while we found no difference in terms of absolute bacterial abundance (GLMM Wald test: $X^2 =$
673 1.08, $P = 0.30$; Fig. 6F), there was a significant difference in their microbiome composition
674 (PERMANOVA with life stage nested within species within genus within subfamily: $R^2 = 0.01$,
675 $F_{3,261} = 1.35$, $P < 0.05$; Table S12), a higher bacterial richness (GLMM Wald test on number of
676 ASVs with firefly genus used as a covariate: $X^2 = 4.99$, $P < 0.05$), and a higher bacterial diversity
677 (GLMM Wald test on Shannon diversity with firefly genus used as a covariate: $X^2 = 7.40$, $P <$
678 0.001) in juveniles compared to adults. These results appear to be driven by the difference
679 between larvae and adults (Supplementary Text 1; Fig. S18).

680 The single larva from the *Photinus* genus, identified through DNA barcoding as from the
681 *Pn. consanguineus* group (Fig. S1), did not appear to have a different bacterial composition
682 compared to adults, and did not harbor any *Williamsoniiplasma* spp. (Fig. S19). In contrast, *Pr.*
683 *versicolor* group larvae had a different bacterial composition compared to adults
684 (PERMANOVA: $R^2 = 0.03$, $F_{1,84} = 2.22$, $P < 0.01$; Fig. S19), including a significantly higher
685 abundance of unidentified *Rhizobiales* (MaAsLin2: coef = 8.92, FDR *P*-adj < 0.05). Notably,
686 one larva specimen from this species group had a higher bacterial titer compared to the two other
687 larvae and harbored *Sp. ixodetis* with high relative abundance. All three *Photuris* larvae analyzed
688 hosted *Leucobacter* in high relative abundance, which was also commonly found in adults (Fig.
689 S9 and S10).
690 Juveniles from the *Py. borealis* species did not have a significantly different bacterial
691 community as a whole when compared to adult males from the same species (PERMANOVA: R^2
692 $= 0.25$, $F_{1,6} = 1.99$, $P = 0.11$; Fig. S19). Nevertheless, and despite our low sample size,
693 significant differences in the abundance of single bacteria across larvae, pupae, and adult males
694 could be noted. The mollicute *Wi. somnilux*, which was commonly detected in *Py. borealis*
695 fireflies, associated with adults and larvae at similar abundances (MaAsLin2: coef = 0.14, FDR
696 *P*-adj = 1; Fig. S20), but was present in lower abundance in pupae (MaAsLin2: coef = 7.92, FDR
697 *P*-adj < 0.01). Other bacterial genera, including *Rhodococcus*, had a significantly higher
698 abundance in *Pyractomena* juveniles compared with adult males (MaAsLin2: coef > 1.99, FDR
699 *P*-adj < 0.05; Fig. S19). These results indicate that juveniles harbor a more diverse microbiome,
700 and some of the most prevalent and abundant adult-associated bacteria are also abundant in
701 larvae and detected in pupae.
702

703 **5. The tissue localization of bacteria varies across firefly species.**

704 We investigated preferential colonization of bacteria in specific tissues of three firefly species
705 and species groups through dissections and tissue separations. We found a significant difference
706 in overall bacterial composition across firefly tissues, but only for unidentified species from the
707 *Pr. versicolor* group, between their lantern and gut tissues (pairwise adonis: $R^2 = 0.12$, $F = 2.36$,
708 FDR P-adj < 0.01; Fig. S21), indicative of major tissue differences only in *Photuris* fireflies.

709 **5.a. *Pn. corruscus* tissues do not differ in bacterial titer but have a few differentially**
710 **abundant bacterial taxa.** Bacterial densities were relatively high throughout the body of *Pn.*
711 *corruscus* (Fig. 7A). In terms of composition, *En. corruscae* commonly colonized all the main
712 tissues of both males and females, suggesting that most *Pn. corruscus* tissues are colonized by
713 bacteria in high abundance, including prevalent mollicutes.

714 Two females had their gut segments and reproductive organs separated through
715 dissection. The mollicute *En. corruscae* was not found in ovaries of these females, but was
716 present in other reproductive organs, and was very abundant in the midgut (Fig. 7A, 7C, 7D and
717 S22). In contrast, *Rickettsiella costelytrae* was very abundant in their carcass, hindgut, and
718 ovaries and was also detected in the genitalia, spermatheca and spermatophore-digesting gland,
719 and midgut of these two individuals, matching infection patterns typical of symbionts residing
720 inside host tissues (i.e., endosymbionts; Perlmutter and Bordenstein 2020). Mollicutes and
721 endosymbionts may thus slightly differ in their tissue localization in *Pn. corruscus*, *En.*
722 *corruscae* being mainly found in the midgut but also infecting reproductive organs, and
723 endosymbionts such as *Rickettsiella* colonizing most tissues, including ovaries.

724 **5.b. Bacteria variably colonize the tissues of *Pn. pyralis* but with a potential primary**
725 **gut localization of *Williamsoniiplasma*.** While there were no significant differences in bacterial

726 titers across *Pn. pyralis* dissected tissues (emmeans: *t*-ratio < 1.93, *P* > 0.22; Fig. 7B), the gut
727 and reproductive organs could occasionally be infected with high titers. When present, *Wi.*
728 *lucivorax* was found to be common and relatively abundant in the gut, and while it was
729 sometimes detected in the carcass and reproductive organs of males, it appeared virtually absent
730 from the lantern (MaAsLin2: coef > 3.79, FDR *P*-adj < 0.05; Fig. 7B and S23). Despite being
731 much less abundant overall than its sister species, the abundance of *Wi. luminosum* was also
732 reduced in the lantern compared to the gut (MaAsLin2: coef = 2.40, FDR *P*-adj < 0.001). This
733 suggests that, as for *Pn. corruscus*, mollicutes may have a primary gut localization in *Pn. pyralis*,
734 despite lower bacterial titers overall in this firefly species.

735 **5.c. Specimens from the *Photuris versicolor* group harbor high density gut bacteria.**
736 While bacterial density in unidentified species from the *Pr. versicolor* group whole body was
737 relatively high (Fig. 1D and S5), it peaked in the gut, especially compared to the lantern and
738 carcass (emmeans: *t*-ratio > 2.85, *P* < 0.05; Fig. 7E and S24). All individuals of these species had
739 a consistently high bacterial titer in the gut, regardless of sex, collection date, and location.
740 Reproductive organs also had significantly higher bacterial titers than the lantern (emmeans: *t*-
741 ratio = 2.71, *P* < 0.05).

742 When separating the unidentified *Pr. versicolor* group specimens by sex, we found that
743 bacteria from the *Curvibacter* genus dominated the microbiome of male guts and reproductive
744 organs compared to female guts and other male tissues (male gut vs. all other tissues except male
745 reproductive organs: MaAsLin2: coef > 4.19, FDR *P*-adj < 0.05; Fig. 7E and Table S11).
746 Otherwise, the gut of both females and males included a high abundance of *Leucobacter* in the
747 gut and reproductive organs compared to the carcass and the lantern (MaAsLin2: coef > 2.93,
748 FDR *P*-adj < 0.05). Notably, one female harbored *Wolbachia* from the supergroup B in all its

749 tissues and another one had a high abundance of *Sp. lampyridicola* in its gut (Fig. 7E). *Photuris*
750 fireflies thus harbor high density gut bacteria with compositions that seem to differ between
751 males and females.
752

753 ## Discussion

754 The firefly family includes relatively understudied taxa when it comes to their microbiome.
755 Here, through our comprehensive characterization of bacterial communities from several
756 Northeastern US firefly species, we studied the factors shaping these insects' microbiomes.
757 Echoing previous research (Green et al. 2021; Hackett et al. 1992), we found high variation in
758 bacterial composition and abundance within Northeastern US firefly species (Fig. 1D, 1E, 4A,
759 S5, S9, and S10). However, we also found that the bacterial community of most firefly species is
760 unique (Fig. 2C–2F), implying that each host species preferentially associates with its own set of
761 bacteria. This high species-specificity combined with intra-specific variability could be
762 explained by the interplay between stochastic and deterministic mechanisms. This was supported
763 by our analysis of normalized stochasticity ratios (NST), showing that either mollicutes or
764 endosymbionts (or both in some *Photuris*) were acquired by fireflies through deterministic
765 factors, but when these symbionts were not included in the microbiome the rest of these firefly-
766 associated bacterial communities were mainly structured through stochastic forces (Fig. 1C).
767 These results highlight the importance of partitioning microbiome analyses based on microbial
768 incidence (e.g., *Mollicutes*) and known functional groups (e.g., endosymbionts), as different
769 ecological processes dictate their acquisition and maintenance by hosts.
770

771 **Ecological mechanisms structuring firefly microbiota**

772 Mollicutes and some endosymbionts may be acquired and maintained through deterministic
773 forces, such as habitat filtering mechanisms, including some component of the host physiology
774 favoring the acquisition, transmission, and establishment of only specific microbes (Mazel et al.
775 2018; Michalik and Szklarzewicz 2025; Ohbayashi et al. 2015; Sachs et al. 2004), and bacteria-
776 driven exclusion or facilitation of the establishment of other bacteria (Alizon, de Roode, and
777 Michalakis 2013; Zélé et al. 2018). Other bacteria composing firefly microbiomes may largely
778 be free-living bacteria acquired from the environment, and their acquisition may depend on
779 stochastic mechanisms, such as random exposure events experienced individually by each firefly
780 (Jones et al. 2022; Zhou and Ning 2017). We further discuss these mechanisms and how they
781 contribute to structuring the firefly microbiome below.

782 First, some of our results support the idea that variability in firefly microbiomes is due to
783 stochastic community assembly mechanisms, including the random acquisition of various free-
784 living microbes from the environment, as suggested before (Green et al. 2021). For example, we
785 found that, for several firefly species sampled in multiple regions, the prevalence of individual
786 bacterial taxa changed across fireflies from different geographic locations (e.g., *Lactococcus* in
787 *Photinus* species; Fig. 5A–5B). A similar variation was observed in *Photuris* microbiomes across
788 habitats with different levels of urbanization (Fig. 6D and S16). This suggests that acquisition of
789 bacteria from environmental pools, which differ in their composition across geographic locations
790 and habitat types (Hosen et al. 2017; Malard et al. 2019; Martiny et al. 2006; Zheng et al. 2024),
791 is probable in fireflies.

792 Second, deterministic habitat filtering mechanisms imposed by host physiological
793 conditions are likely to participate in shaping the establishment of characteristic members of the
794 bacterial communities. Supporting this, we found that, despite being collected at the same

795 locations, times of day, and seasons, sympatric firefly species (Faust 2017; Lloyd 2018; Martin
796 et al. 2024; Stanger-Hall and Lloyd 2015) did not share similar microbiomes, whether they were
797 closely related or not (Fig. 2A–2F). As previously pointed out by Hackett et al. (1992), even
798 fireflies from the *Photuris versicolor* group, which consume *Photinus* and *Pyractomena* fireflies,
799 did not harbor any of the *Williamsoniiplasma* bacteria characteristic of their prey (Fig. 4A and
800 4B). In addition, several firefly-associated bacterial taxa from the *Mollicutes* may be endemic to
801 fireflies since they have only been found in firefly tissues to date (Fig. 3). These results are
802 indicative of host restriction, suggesting that fireflies are not entirely permeable to the microbes
803 they are exposed to, and their microbiome composition cannot be fully explained by passive
804 colonization of environmental bacteria. Unknown habitat filtering mechanisms within the host
805 are thus likely to contribute to the assembly of firefly microbiomes, as is the case in other
806 organisms (Ohbayashi et al. 2015; Sachs et al. 2004).

807 Vertical transmission can be considered as a special case of deterministic host filtering
808 mechanism, where only specific symbionts are transmitted from parents to offspring and
809 conserved by the host line throughout generations. Many insects associating with lineage-
810 specific, primary symbionts have been shown to rely on strict vertical transmission to preserve
811 key beneficial functions from the symbiont (Baumann 2005; McCutcheon, McDonald, and
812 Moran 2009; Sacchi et al. 2000). Yet, several endosymbionts that are common across arthropods,
813 such as *Rickettsia*, *Rickettsiella*, *Spiroplasma*, and *Wolbachia* practice a mix of vertical and
814 horizontal transmission with no strict host specificity (Gu et al. 2023; Liu et al. 2024; Michalik
815 and Szklarzewicz 2025; Perreau and Moran 2022). Here, we confirmed the endosymbiotic nature
816 of *Rickettsiella* and *Wolbachia* in fireflies, which infected several tissues, including reproductive
817 organs (Fig. 6A–E). While our NST analysis suggests that the acquisition of these

818 endosymbionts was deterministic in *Pn. marginellus* group and unidentified species from the *Pr.*
819 *versicolor* group (Fig. 1C), these endosymbionts appeared with a more random distribution in
820 other firefly species (Fig. 4A), suggesting a stochastic influence, perhaps conditioned by
821 horizontal jumps across hosts. Although we detected endosymbionts and mollicutes in female
822 reproductive organs (Fig. 6A), it is still unclear whether they are vertically transmitted to firefly
823 offspring.

824 Third, bacteria-driven exclusion or facilitation is another deterministic process that could
825 structure firefly-associated bacterial communities. In insect microbiomes, some bacteria have to
826 compete to establish in a host (Itoh et al. 2019), while others need other bacterial species to be
827 present (Carpenter et al. 2021). We found that when they were detected in fireflies, *Rickettsia*
828 and *Wolbachia* were likely to dominate bacterial communities and rarely co-occurred with other
829 bacteria (Fig. S11). This result could be a consequence of potential competitive exclusion driven
830 by these known endosymbionts (Caragata et al. 2013). However, we cannot exclude the
831 possibility that these endosymbionts were present at such high densities in firefly tissues that the
832 sequencing depth was not enough to detect other co-occurring bacteria (Gofton et al. 2015). We
833 note that *Rickettsia bellii* and *Wolbachia* supergroup B usually had a very high absolute bacterial
834 abundance in specimens (Fig. S19 and S24), supporting this latter possibility. We also found
835 patterns of positive co-occurrence among bacteria associated with a few firefly species, which
836 could suggest facilitation. Chief among them is the *Pseudomonas* and *Stenotrophomonas* duo
837 found in several *Photinus* fireflies (Fig. S11), also known to cooperate during infections of
838 vertebrates (McDaniel et al. 2023; McDaniel, Schoeb, and Swords 2020). In *Photuris* fireflies,
839 *Curvibacter*, *Achromobacter*, and *Elizabethkingia* tend to co-occur in the reproductive organs
840 (Fig. 7E) and in the gut (Green et al. 2021), suggesting acquisition either from sexual

transmission or from food intake. Future work should investigate whether these bacteria are acquired together by chance (Carpenter et al. 2021), or truly facilitate each other's establishment in firefly hosts (Halliday et al. 2024; Zélé et al. 2018).

It thus appears that, as in many animals (Chandler et al. 2011; Furman et al. 2020; Hendrycks et al. 2025; Oliphant et al. 2019), fireflies acquire their microbiomes through a combination of stochastic and deterministic elements. Mechanisms involved likely include the acquisition of environmental bacteria, habitat filtering mechanisms—including potential reliable transmission modes, partner choice processes to select specific bacterial strains, and/or parasitic associations where parasites circumvent barriers to establishment—and possible competition and facilitation among bacterial strains. Future studies should test the contribution of each of these stochastic and deterministic mechanisms in structuring firefly microbiota.

Which factors influence the association with bacterial symbionts?

As a group, fireflies offer diverse ecologies across species and throughout their lifetime. We investigated the influence of several firefly traits, such as sex, dietary ecology, and life stage, on the composition and abundance of housed bacteria. Notably, we did not find convincing evidence linking adult bioluminescence to bacterial symbiosis, since bioluminescent firefly species had similar bacterial loads and composition as non-bioluminescent ones (Fig. S12) and the lantern of two common species housed either low or variable bacterial density with seemingly random bacterial composition, except in the rare occasion of infections with an endosymbiont colonizing all tissues including the lantern (Fig. 7B, 7E, S23, and S24).

Interspecific variation in the animal microbiome is often influenced by diet (Degregori et al. 2024; Karasov and Douglas 2013). Here, we found that bacterial density and composition

864 changed between fireflies with adults known to eat versus those with supposed non-eating adults
865 (Fig. S12). However, since diet and phylogeny are confounded, this result was not well
866 supported when accounting for host phylogenetic relationships, which were responsible for a
867 large portion of microbiome (dis)similarity among firefly species (Fig. 2A–C, and S7). Notable
868 microbiome divergences observed between closely related firefly species could, nevertheless, be
869 attributed to species' differing ecology, perhaps including dietary differences. For example, *Pn.*
870 *pyralis* and *Pn. corruscus*, which are two closely related species (Catalan et al. 2022) with
871 overlapping geographic range—the former not seemingly eating as adults and having a short
872 adult lifespan from May to August, and the latter consuming plant sap and nectar and living from
873 November to May as adults—harbored divergent bacterial densities and compositions (Fig. 1D
874 and 2F). In-depth dietary analyses should be performed to formally address the contribution of
875 adult diet to microbial abundance and composition in fireflies, while controlling for host
876 relatedness.

877 Our results suggest that some of the intraspecific variation in bacterial abundance in the
878 winter firefly *Pn. corruscus* could be explained by seasonal effects. We found that the abundance
879 of the mollicute *Entomoplasma corruscae* associated with these fireflies increased in the spring
880 (Fig. 6B), when reproductive activity peaks for this firefly species in the Northeastern US (Fig.
881 6C). Given that this bacterium was sometimes abundant in the midgut, but could also be detected
882 in the reproductive organs and carcass (Fig. 7A–7D, and S22), several possible modes of
883 acquisition may promote the proliferation of *En. corruscae* in the spring. First, *Pn. corruscus*
884 fireflies may drastically increase food intake during the reproductive season (Rooney and Lewis
885 2000), hence acquiring large quantities of this bacterium from their food sources, thus implying
886 an environmental acquisition mode. But it is also possible that *En. corruscae* is vertically

887 transmitted from parents to offspring, although this was not supported in a recent study analyzing
888 the bacterial composition of six eggs (Green et al. 2021). Nevertheless, this bacterium could
889 potentially reside inside their hosts with very low, barely detectable, abundances at the egg,
890 larval, pupal stages, and in overwintering adults, and begin to replicate as endogenous hormonal
891 pathways are triggered by reproductive maturity and/or through warming temperatures, like in
892 other insects (Liu et al. 2024; Poulain et al. 2024; Smith et al. 2021). Finally, as seen in the
893 association between aphids and three facultative endosymbionts (Moran and Dunbar 2006), *En.*
894 *corruscae* could potentially be sexually transmitted (i.e., horizontal transmission), and its
895 abundance in adult individuals could be directly proportional to the number of copulation events
896 they experience.

897 As holometabolous insects, fireflies have two dissociated, active life stages with
898 drastically divergent ecologies, translating into distinct periods of exposure to, and interaction
899 with, microbes (Hammer and Moran 2019; Manthey et al. 2023): one at the larval stage, after
900 eggs hatch, and one at the adult stage, following pupation. While the ecology, physiology, and
901 behaviors of adult fireflies have been well documented, earlier developmental stages (e.g., eggs,
902 larvae, pre-pupae, and pupae) remain understudied, largely due to the cryptic lifestyles of these
903 early stages (e.g., soil dwelling) and difficulties to rear them throughout their development
904 (Riley, Rosa, and Lima da Silveira 2021). However, it is crucial to study larval microbiomes,
905 since fireflies spend most of their life in this stage. Here, we found that firefly larvae from the
906 Northeastern US harbored a more diverse bacterial community compared to adults (Fig. 6F and
907 S18), with diversity index values and a few bacterial genera similar to those from a study on the
908 gut microbiota of distantly related, *Aquatica leii* firefly larvae from China (Zhao et al. 2023).
909 When focusing on key bacterial groups in fireflies, although our single *Pn. consanguineus* group

910 larva did not harbor any mollicute in high abundance, we found that all five late stage *Py.*
911 *borealis* larvae, sampled in different locations (northern Delaware, eastern New York,
912 southeastern Pennsylvania), harbored moderate to high abundance of the mollicute *Wi. somnilux*
913 (Fig. S19 and S20). While the high diversity of larva-associated bacterial communities could
914 easily be explained by environmental acquisition of diverse bacteria from the microbe-rich
915 habitats firefly larvae occur in, such as soil (Torsvik, Goksøyr, and Daae 1990), the presence of
916 the same mollicute species in *Py. borealis* larvae collected from locations separated by hundreds
917 of kilometers further supports our finding that deterministic mechanisms promote the acquisition
918 and persistence of species-specific mollicutes in fireflies. However, it is still unknown whether
919 this mollicute species is acquired from the specific environment of *Py. borealis* larvae.
920 Transmission experiments should be performed in this firefly species to determine the bacterial
921 mode of transmission.

922 Another mystery lies in the fate of symbionts through metamorphosis to adulthood in
923 holometabolous insects. Here, we found that one *Py. borealis* pupa (which was approaching
924 emergence) out of three had detectable, yet low, levels of *Wi. somnilux* (Fig. S19 and S20). This
925 bacterium has originally been sampled multiple times in the pupal gut of *Py. angulata* from
926 Maryland (Hackett et al. 1992; Williamson et al. 1990). It thus appears that, at least in
927 *Pyractomena* fireflies, larvae, pupae, and adults associate with species-specific mollicutes, which
928 occasionally appear to persist in the gut during pupation. While a drastic reduction in abundance
929 of larva-associated microbes is common during the metamorphosis of holometabolous insects
930 (Hammer and Moran 2019), the retention of symbionts through pupation has been shown in a
931 few insects before. For example, the fruit fly *Drosophila melanogaster* can harbor high
932 abundance of the mollicute *Spiroplasma* in the gut throughout its lifetime, including during

933 pupation (Anbutsu and Fukatsu 2003). The possible retention of species-specific mollicutes
934 through pupation is yet another indication of the tight, non-random association between these
935 bacteria and their firefly hosts.
936

937 **Insights into the evolutionary history of firefly-associated mollicutes**

938 While the transmission mode of mollicutes in fireflies is still uncertain, we found support that
939 these bacteria non-randomly associate with firefly hosts. Although this is not a prerequisite for
940 being a symbiont specialist (Bourguignon et al. 2018; Cabuslay et al. 2024; Koch et al. 2013),
941 several ancient symbionts are so dependent on their host that they have co-diversified with it
942 (García-Lozano et al. 2024; Hu et al. 2023; Kikuchi et al. 2009; Lo et al. 2003; Zhang et al.
943 2023), forming patterns of co-phylogeny where the symbiont phylogeny tightly matches that of
944 their host. Our phylogenetic analyses (Fig. 3 and S3), along with previous phylogenomic results
945 (Gupta et al. 2019; Lo et al. 2018), suggest limited evidence for co-cladogenesis between the
946 *Mollicutes* and fireflies, since mollicutes infecting closely related firefly species are rarely each
947 others' closest relatives. For example, the closely related fireflies *Pn. corruscus* and *Pn. pyralis*
948 (Catalan et al. 2022) commonly harbored *En. corruscae* and *Wi. lucivorax*, respectively,
949 classified in two distinct bacterial genera (Gupta et al. 2019). These two bacterial species are
950 phylogenetically separated by other bacterial genera with very different lifestyles (e.g., members
951 of the *Mycoplasma* are vertebrate pathogens; Fischer et al. 2012; Waites and Talkington 2004).
952 Even within the mollicute genera that infect fireflies, the preferred host can vary. For example,
953 the *Williamsoniiplasma* genus includes species associated with either lampyrine fireflies or tiger
954 beetles (Tully et al. 1998), which last shared a common ancestor over 300 million years ago
955 (McKenna et al. 2019). Thus, it appears unlikely that mollicute symbionts are the result of

956 ancient domestication in a common host ancestor and conserved through firefly evolution to
957 extant lineages.

958 This incongruence between host and symbiont phylogenies could have two explanations.
959 First, species from the *Mollicutes* may be generalists and associated with multiple host species.
960 These bacteria could switch hosts on multiple occasions over evolutionary time scales, a process
961 that has been shown in other insect-symbiont systems (Sudakaran, Kost, and Kaltenpoth 2017).
962 While most *Williamsoniiplasma* species appear to specifically associate with one lampyrine
963 species or genus (Fig. 4A), the *Wi. luminosum* species was detected at moderate to high
964 abundance in three *Photinus* firefly species (Fig. 4A and S9; Fallon et al. 2018; Williamson et al.
965 1990), suggesting a lower level of host specificity for this mollicute species. This is also
966 supported by *En. ellychniae*, a bacterial strain with a 16S rRNA amplicon sequence almost
967 identical to *En. corruscae* (Fig. 3), being able to infect *Pn. corruscus* as well as tabanid flies
968 (Wedincamp et al. 1996); and by members of the *Spiroplasma* genus, with single *Spiroplasma*
969 species being detected in different insect species (Hackett et al. 1992, 1996; Stevens et al. 1997;
970 Tully et al. 1995). Host switching could conceivably be facilitated by interspecific sexual
971 interactions, a behavior that has been documented among *Photinus* fireflies (Vergara, Maquitico,
972 and Cordero 2025). Future laboratory transplantation experiments could help discern the extent
973 of species-specificity in firefly-mollicute associations.

974 Another possible explanation for the discrepancy in firefly-mollicute phylogenies is that
975 some of these associations may have evolved recently, leading to patterns of co-phylogeny that
976 may only be detectable at lower taxonomic levels, within single mollicute genera, across species
977 or strains. The *Entomoplasma* and *Williamsoniiplasma* genera, for example, may include
978 additional firefly-associated symbiont species that have to be discovered from yet unscreened

979 firefly species. Given the endemism of *Wi. lucivorax* to *Pn. pyralis* fireflies, other unscreened
980 lampyrine species could plausibly harbor undiscovered, endemic mollicute taxa. The microbiome
981 of more firefly species should be characterized to determine whether they harbor new mollicute
982 species, sisters to the ones that have already been identified.
983

984 **Conclusion**

985 Through qPCR, deep amplicon sequencing, dissections, phylogenetics, and bioinformatic
986 analyses, we characterized the bacterial communities of fireflies from two common subfamilies
987 in the Northeastern US and evaluated the influence of different factors on bacterial community
988 structure and composition. We found that most firefly species harbor a unique, yet variable
989 microbiome, structured by a combination of stochastic and deterministic forces, and often
990 dominated by members of species-specific mollicutes. Although the intraspecific variation
991 observed in firefly microbiomes likely derives from a combination of stochastic process and
992 geographic, environmental, and host-specific factors, like life stage, age, and sex, the non-
993 random preferential associations with members of the *Mollicutes* raises the possibility that host-
994 specific physio-chemical conditions (i.e., habitat filtering mechanisms) contribute to the
995 maintenance and specificity of these bacteria. While the transmission mode of these bacteria is
996 still unknown, given their high abundance when present, mollicutes may play a role in the fitness
997 of firefly hosts (Supplementary Text 1). We also occasionally found insect endosymbionts
998 infecting firefly tissues, with yet undetermined roles, but with a hypothetical potential for
999 reproductive manipulation.

1000 Altogether, our results suggest that fireflies are associated with species-specific bacteria
1001 that are not always present, and that may be facultative symbionts. In the future, functional

1002 characterization of firefly microbiomes through a combination of targeted experimental
1003 antibiotic and fitness assays, genomics, and gene expression analyses will be necessary to better
1004 comprehend the nature of the microbe-firefly associations. Firefly biology and ecology can be
1005 challenging to study, given the short adult lifespans of most species and the difficulty to rear
1006 early developmental stages, especially terrestrial species, in laboratory settings. Improvements of
1007 existing methodologies (Buschman 1988; Fallon et al. 2018; Fu and Benno Meyer-Rochow
1008 2013; Ho et al. 2010; McLean, Buck, and Hanson 1972; Vaz et al. 2020; Yang et al. 2024) could
1009 facilitate the study of their microbiome. This research constitutes a major first step towards a
1010 better understanding of the ecology of firefly-associated microbial communities. Future research
1011 will shed light on how microbes contribute to the fascinating but puzzling ecology and evolution
1012 of fireflies, a group that provides pest control services (Fu and Benno Meyer-Rochow 2013) and
1013 has high aesthetic value, but is experiencing severe population reductions around the world
1014 (Fallon et al. 2018; Lewis et al. 2020).
1015

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1023

1024 ## Author contributions

BB designed the study, performed the dissections, analyzed the data, and wrote the initial manuscript. BB and SEL collected specimens. BB, SRN, and AR extracted DNA. BB and TJD performed DNA barcoding. BB and AR processed the amplicon sequencing data. BB, SEL, and AR contributed to review and editing of the manuscript. All authors read and approved the final version of the manuscript.

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Data availability

All 16S rRNA gene amplicon sequencing data have been deposited in the NCBI Short Read Archive under BioProject PRJNA1237523. The COI DNA barcoding sequences produced from Sanger sequencing and highlighted with orange labels in Fig. S1 are available from the NCBI GenBank database with accession numbers PV844642–PV844700. Supplementary data can be found in our data repository at: https://doi.org/10.6084/m9.figshare.28306616.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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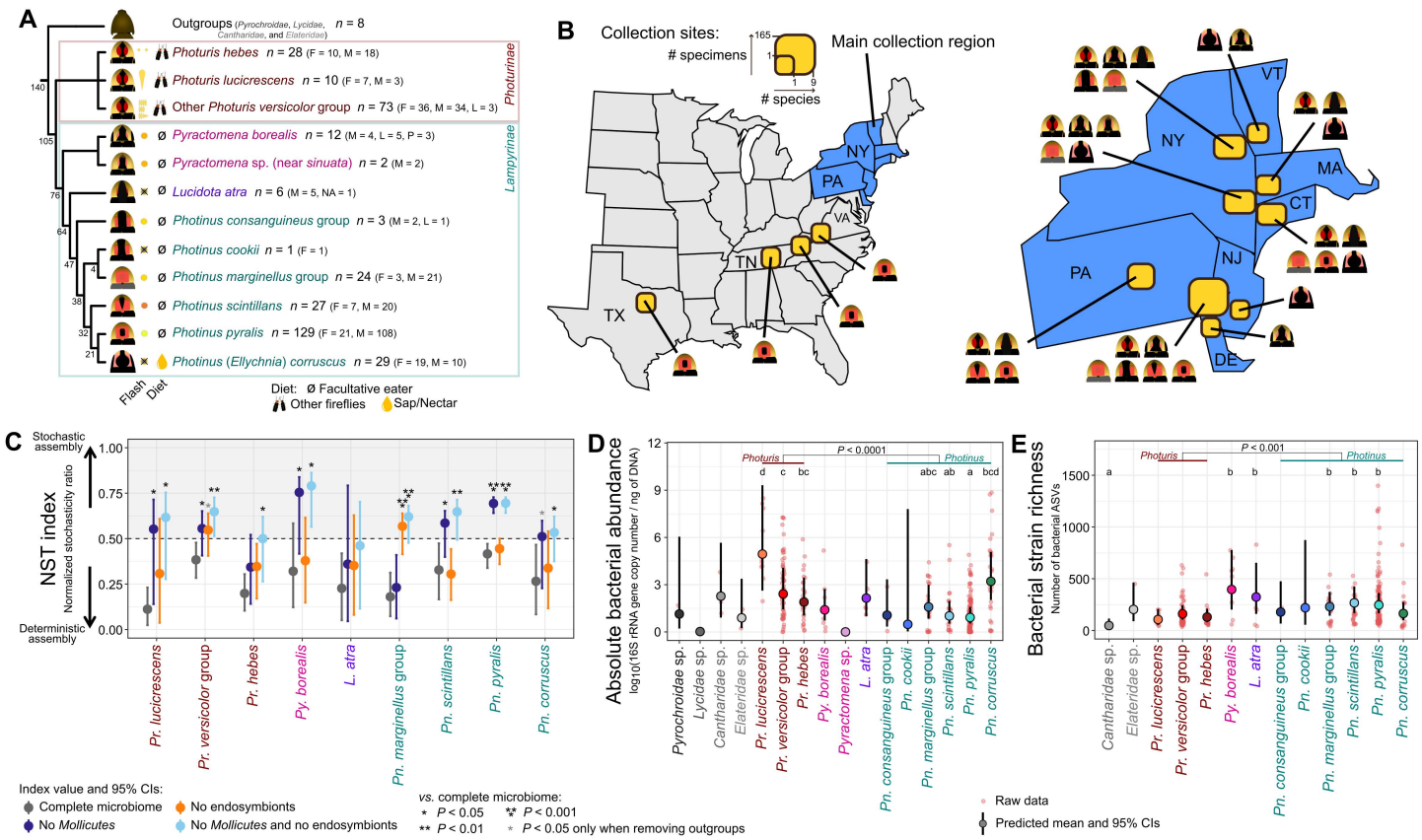

Figure 1: Sampling overview and firefly microbiome abundance and diversity across species. **A**. Species sampled and analyzed in the present study. The phylogenetic tree is adapted from Catalán et al. (2022) and Martin et al. (2019). Numbers at each node indicate estimated divergence time from Catalán et al. (2025), Höhna et al. (2024), and Powell (2022) in millions of years. The cartoons next to the tree branches are simplified representations of the typical markings visible on the pronotum of different firefly species. Symbols next to these pronotal depictions correspond to the most common male flash patterns for the species or species group, with the frequency of the flashes for *Photuris* species, and whether a firefly is bioluminescent (colored flash) or not (crossed out). The next symbols correspond to adult diets, with "ø" for species that are not known to eat as adults, other fireflies for predatory diet, and a yellow droplet for sap and nectar drinkers. The number of specimens collected and processed is shown next to species' names, with "F" for females, "L" for larvae, "M" for males, and "P" for pupae. **B**. Map of the central and eastern United States and Northeastern states showing the location of sampling sites. While a few *Pn. pyralis* specimens were collected in Tennessee, Texas, and Virginia, most fireflies were collected in Northeastern states (blue color). The yellow rectangles give the number ("#") of species (width) and specimens (height) collected in the region and the cartoons of firefly pronotum indicate which species were collected, corresponding to the cartoons from panel A. Specimen information can be found in Table S1. **C**. Normalized stochasticity ratio (NST) across firefly species. NST index values higher than 0.5 indicate a higher importance of stochastic forces governing bacterial community assembly, whereas values lower than 0.5 point to deterministic forces as the main drivers of community assembly. For each firefly species or species group the balls correspond to the index value and the error bars are 95% confidence intervals obtained from bootstrapping. Dark grey color is for the complete, untouched microbiome, dark blue corresponds to microbiomes where all the mollicutes were removed from the dataset, orange is for microbiomes where putative endosymbionts (*Candidatus* Hepatincola, *Rickettsia*, *Rickettsiella*, and *Wolbachia*) were removed, and light blue corresponds to microbiome datasets where both mollicutes and endosymbionts were removed. The asterisks depict, for each species, significantly different NST values between the complete microbiome and either of the modified microbiome datasets. Two-tailed Wilcoxon signed-rank statistical tests were used to test the significance of these differences. Asterisks in light grey are for tests run on datasets where outlier data points (i.e., values higher or lower than 1.5-fold of the interquartile range) were removed. Values and statistical test results can be found in Table S6. **D**. Normalized absolute

bacterial abundance across all firefly and related insect samples. Values are expressed as the $\log_{10}$ of the number of 16S rRNA gene copies in a sample, measured through qPCR, minus the number of 16S rRNA gene copy in the DNA extraction blank sample from the same batch, and then divided by the total DNA concentration in the sample, which was measured via Qubit. Least-square means (emmeans) post-hoc tests on generalized linear mixed models (GLMMs), were used for statistical testing of differences between genera and species, and controlled for sex, day of year, month of capture, time of capture, longitude at capture, local sampling point, and whether the specimen was dissected or not. Lowercase English letters indicate the significance of the difference across firefly species. Letters for species that are not significantly different from any other species (i.e., $P > 0.05$: "abcd") were removed. Balls and error bars correspond to predicted average and 95% confidence intervals obtained from the GLMM. Dots represent raw data points. **E**. Bacterial richness, expressed as the number of bacterial ASVs, across firefly species. The *Photinus* microbiome contains, on average, a higher bacterial strain richness than that of *Photuris* (emmeans post-hoc tests on GLMMs, controlling for genus, sex, local sampling point, day of year, year of capture, and sequencing type). See description of panel D for details on graph components. All alpha diversity metrics can be found in Table S7 and Fig. S6.

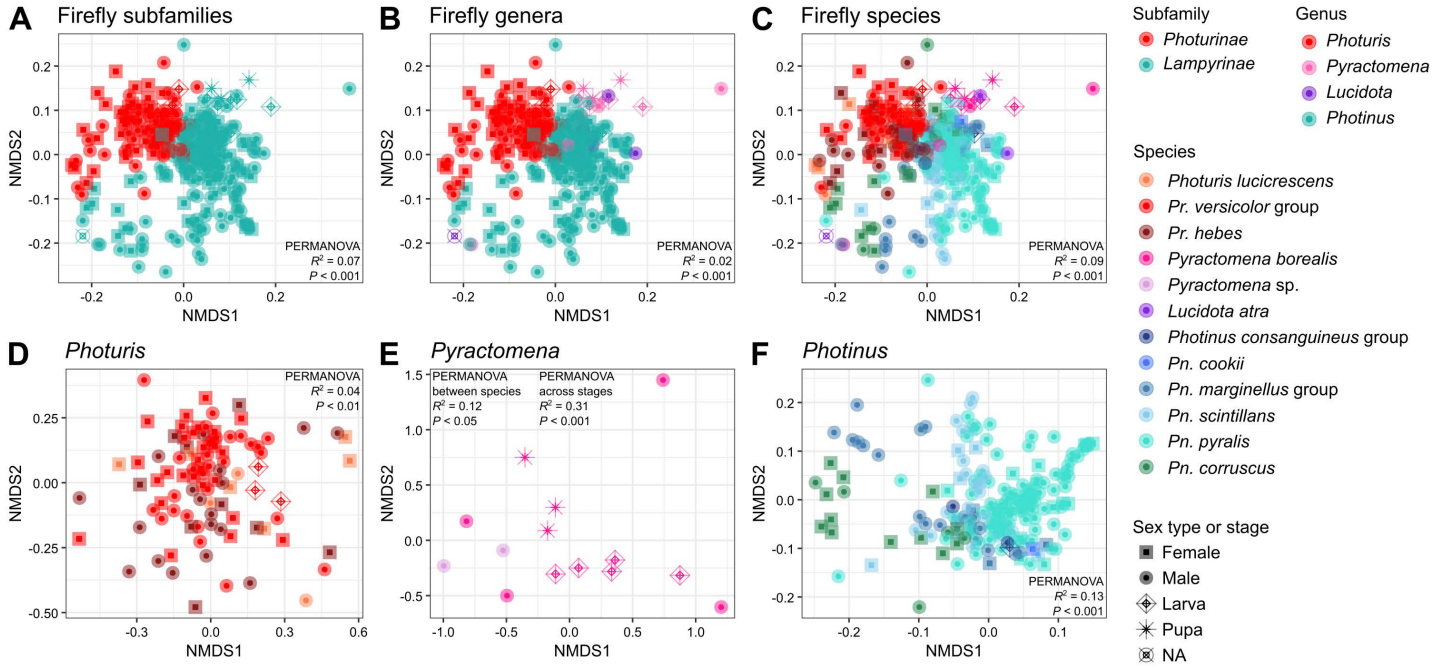

Figure 2: Microbiome divergence across fireflies at different taxonomic levels. **A**, **B** and **C**. Non-metric multidimensional scaling (NMDS) analyses showing separation of the bacterial communities based on composition and relative abundance at the firefly subfamily, genus, and species levels. Four outgroup specimens were removed from these analyses. **D**, **E**, and **F**. NMDS analyses showing variation in the microbiome across species within three firefly genera. All plots are derived from Bray-Curtis distances calculated on non-rarefied, proportional (i.e., compositional) relative abundance of taxon-aggregated bacteria in firefly specimens. Permutational multivariate analyses of variance (PERMANOVAs) were used for statistical testing of group similarities. Nested PERMANOVAs were used for B and C, with genus nested within subfamily and species nested within genus within subfamily, respectively. In panels D and F, the species term was the main predictor in the PERMANOVA models. PERMANOVAs were computed on read depths rarefied at 30,000 reads, except in E where it was computed on non-rarefied data to be able to compare the two *Pyractomena* species (*Pyractomena* sp. near *sinuata* specimens are filtered out by rarefying) or three developmental stages.

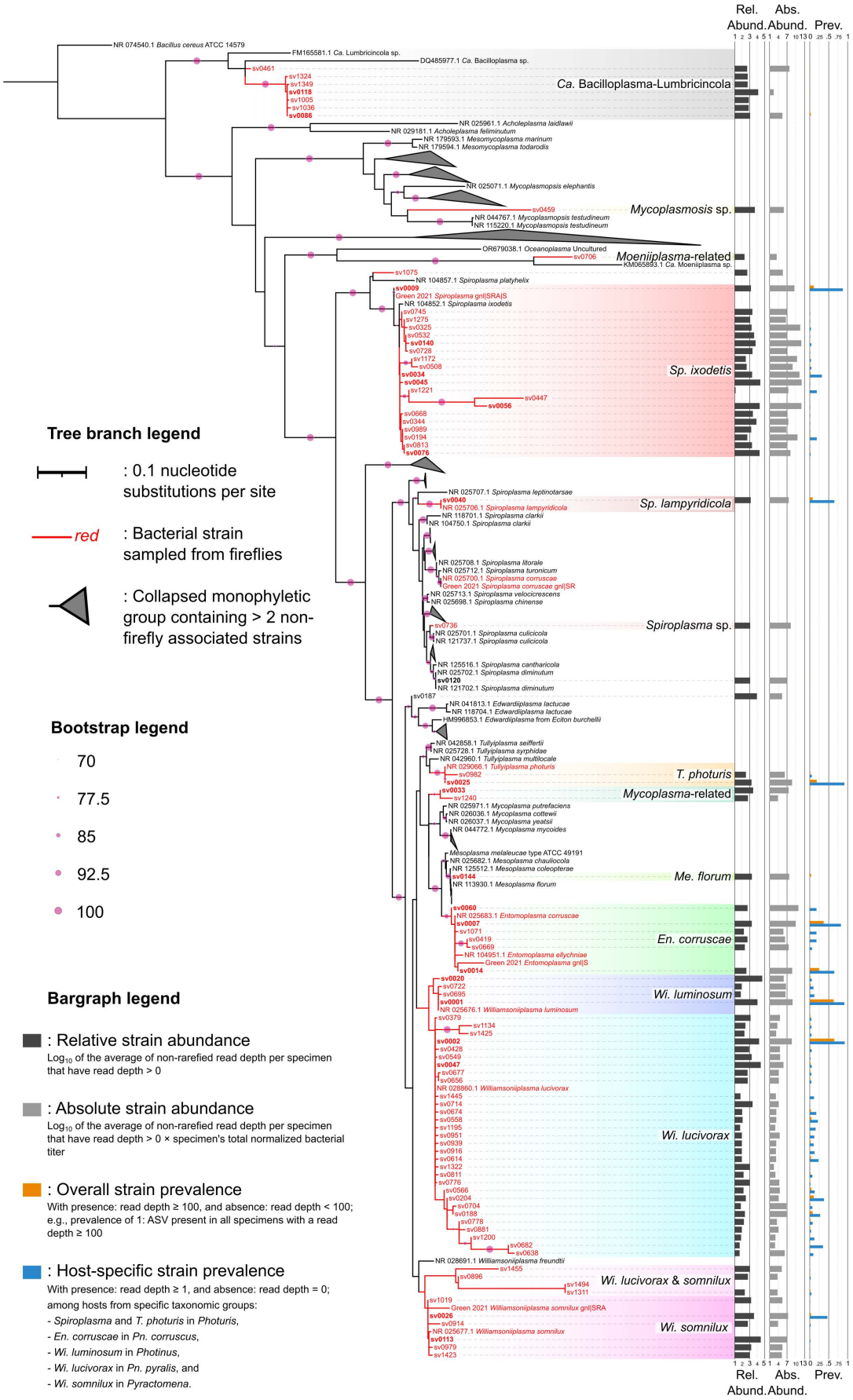

Figure 3: Phylogenetic strain diversity of the *Mollicutes* associated with fireflies. The tree was obtained from a maximum likelihood phylogenetic analysis on the 16S rRNA gene, rooted with a *Bacillus cereus* sequence (NR_074540.1), and contains 230 leaves of which 88 are 16S rRNA amplicon sequences from the

present study, four are amplicon sequences from another firefly microbiome study (Green et al. 2021), and four are amplicon sequences from another study on insect-associated mollicutes (Funaro et al. 2011). All the other leaves are full length 16S rRNA gene sequences and were obtained either through NCBI searches to acquire reference sequences of mollicute species or through NCBI BLASTn runs to find the most similar sequences to our amplicon sequences. Red branches contain bacterial ASVs sampled from fireflies (from the present and previous studies), only focusing on our 1,500 most abundant ASVs (SV identifier number lower than 1,501). ASV labels in bold are among the most abundant in our dataset (SV identifier number lower than 151). Gray triangles correspond to collapsed monophyletic groups containing more than two non-firefly associated leaves. Bootstrap values above 70 are depicted with a pink dot on the tree nodes. Genus and species names are from Gupta et al. (2018, 2019). A detailed version of the tree with uncollapsed leaf labels is shown in Fig. S3. Bargraphs on the right represent ASV average relative abundance ("Rel. Abund.") when present (dark gray), average absolute abundance ("Abs. Abund.") when present (lighter grey), and prevalence ("Prev.") both across all specimens in our dataset (gold) and within specific firefly groups (blue). Only specimens' whole body was considered for these calculations.

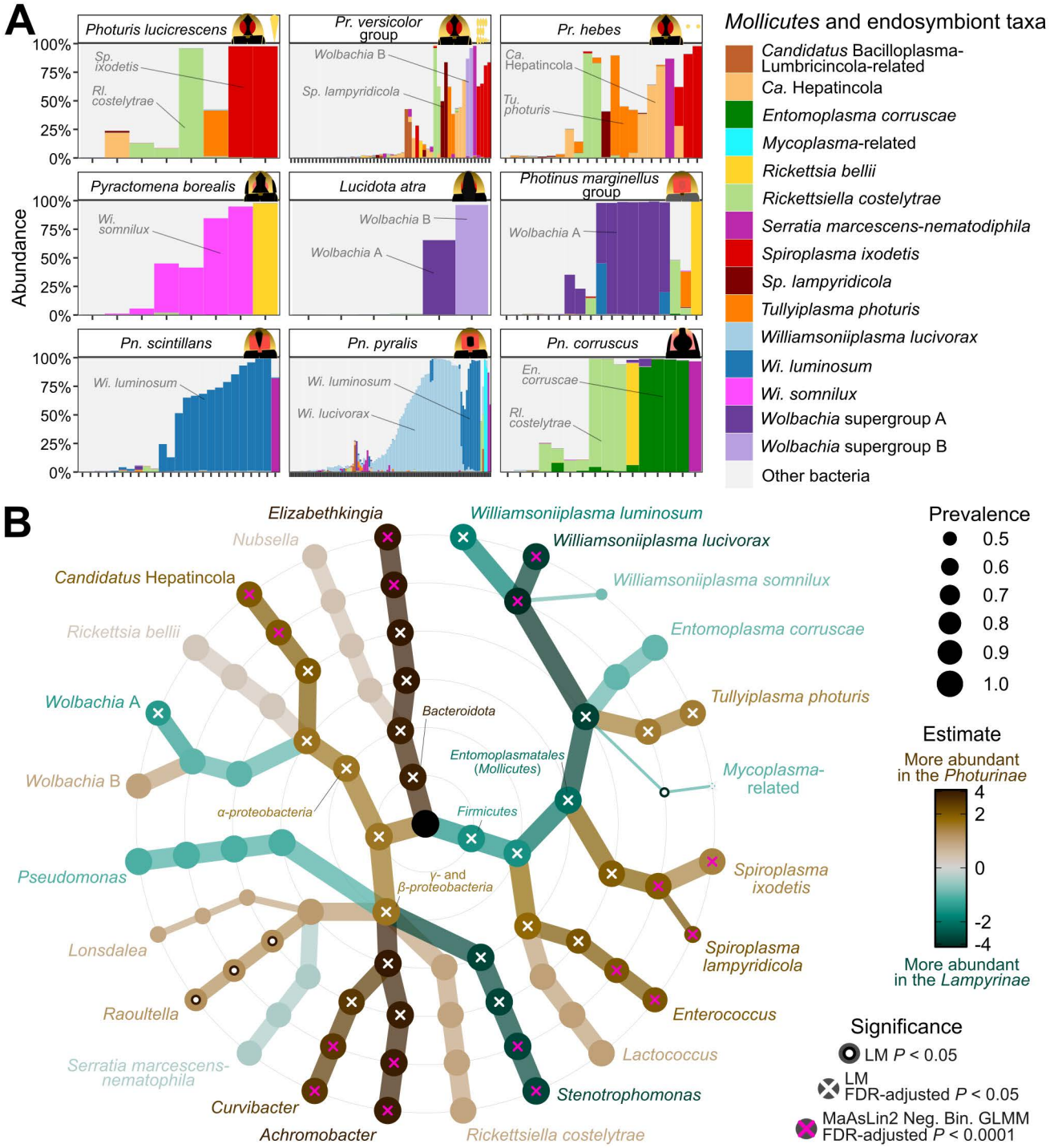

Figure 4: Bacterial composition and differential abundance. **A**. Composition of bacterial taxa across firefly species and species groups with more than three specimens in our dataset post rarefying. Each vertical bar (x-axis) represents one firefly specimen. The relative rarefied abundance (y-axis) of the most abundant mollicutes and putative endosymbionts is shown as different colors whereas the abundance of all other bacteria is in light grey and can be seen in Fig. S8. **B**. Differential bacterial abundance at multiple taxonomic levels across adult fireflies. Only firefly species with more than one specimen in our dataset, post rarefying, and only bacterial taxa with rarefied abundance > 20,000 reads in one or more samples were used in this analysis. Moving outwards from the center, light gray rings show phylum, class, order, family, and genus (sensu SILVA database); bacterial "taxa" are found on the outermost ring of the tree. The size of the balls depicts prevalence of a clade across samples (proportion of firefly specimens in which it was detected). Results from three statistical tests comparing bacterial abundances in *Lampyrinae vs. Photurinae* subfamilies are displayed. Following the replacement of zeros by half of the minimum value (to reduce zero-inflation and improve model fitting) and

$\log_2$ transformation, a linear model ("LM") was run on all bacteria at every taxonomic rank using the *microViz* R package. White circles and crosses on balls indicate significantly differential abundance with unadjusted and false discovery rate ("FDR")-adjusted, respectively, at $P < 0.05$. Estimates of bacterial differential abundance from this LM are displayed with shades of brown (darker: higher abundance in *Photurinae*) and sea green color (darker: higher abundance in *Lampyrinae*). At both the bacterial genus and taxon levels, differential abundance was additionally tested through *MaAsLin2* multiple generalized linear mixed models ("GLMM") with negative binomial ("Neg. Bin.") distribution and controlling for date of capture, geographic origin, and sex. Pink crosses show the bacterial taxa that were found to be significantly differentially abundant at FDR-adjusted $P < 0.001$.

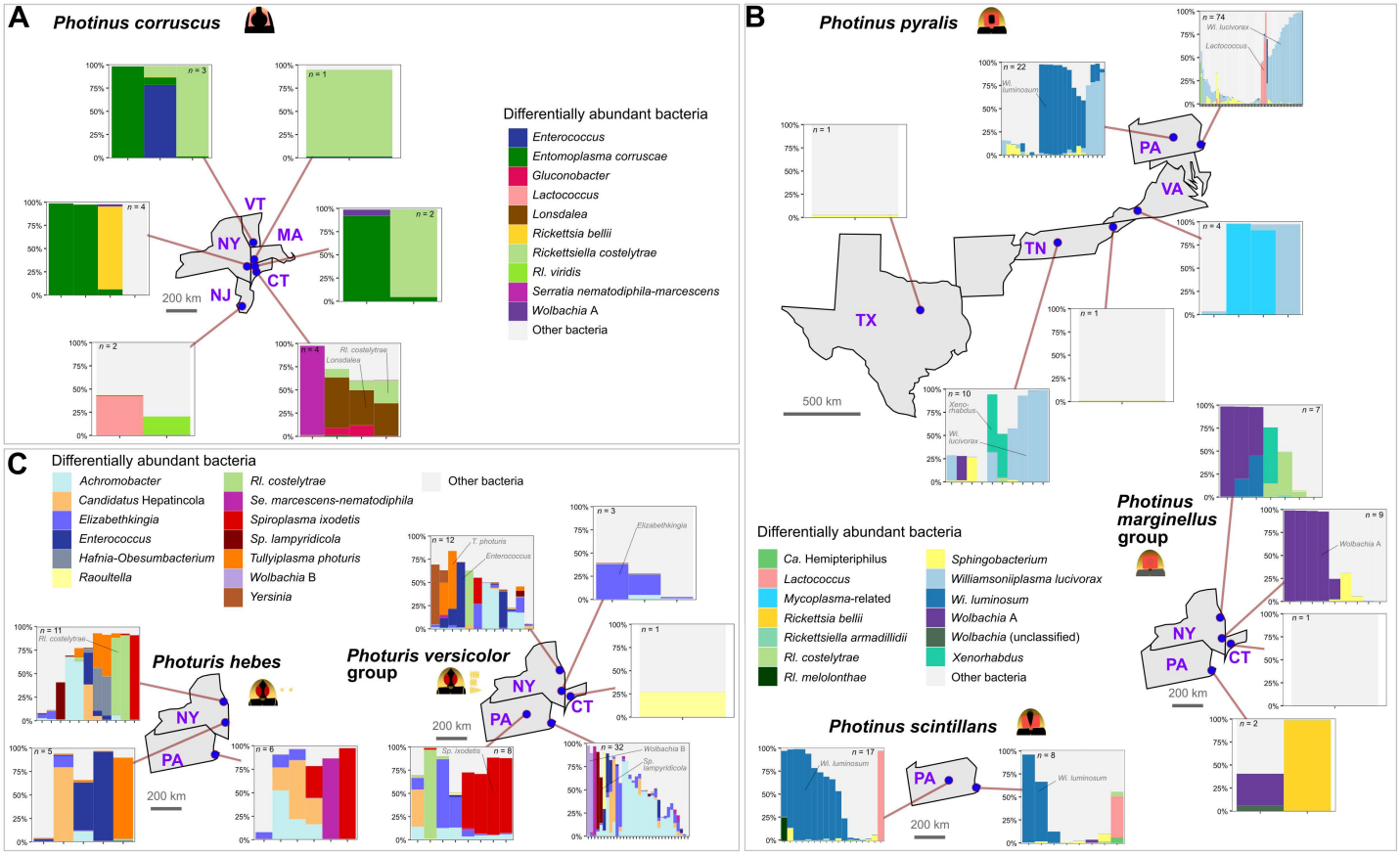

Figure 5: Regional differences in bacterial composition. Bacterial composition, with a focus on the most differentially abundant bacterial taxa across regions. Firefly species are grouped by microbiome similarity, with *Photinus corruscus* in **A**, *Pn. pyralis*, *Pn. marginellus* group, and *Pn. scintillans* in **B**, and *Photuris hebes* and unidentified species from the *Pr. versicolor* group in **C**. Bacterial taxon composition and abundance per species per region was obtained from rarefied read depth. The number of specimens is given within each bar plot. In all panels, "CT": Connecticut, "PA": Pennsylvania, "MA": Massachusetts, "NJ": New Jersey, "NY": New York, "TN": Tennessee, "TX": Texas, "VA": Virginia, and "VT": Vermont US states. A full list of differentially abundant bacterial taxa across regions can be found in Table S9.

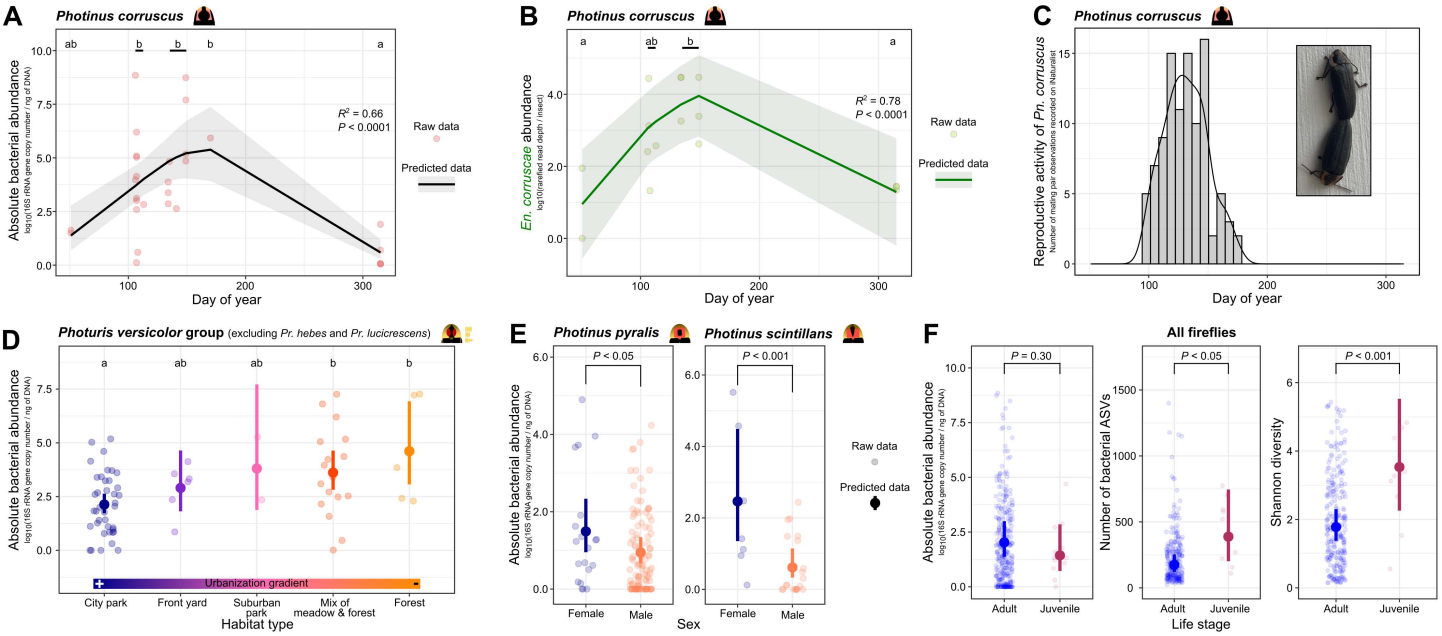

Figure 6: Seasonal, environmental, and life history factors influence the microbiome of fireflies. **A**. Normalized absolute bacterial abundance in *Photinus corruscus* peaks at the end of spring. A generalized linear mixed model (GLMM) was used to test whether bacterial titer is influenced by day of year, used as a quadratic term, in *Pn. corruscus*, when controlling for sex, latitude, year of capture, and whether the specimen was dissected or not. Change in bacterial titers over the months for this species was also tested with least-square means (emmeans) post hoc test on a GLMM controlling for the same factors. Significant differences between February, April, May, June, and November are shown with lowercase English letters. **B**. Abundance of *Entomoplasma corruscae* in *Pn. corruscus* also peaks at the end of spring. *En. corruscae* abundance is expressed as the $\log_{10}$ of rarefied read depth. A GLMM was used to test whether *En. corruscae* abundance is influenced by day of year, used as a quadratic term, in *Pn. corruscus*, when controlling for sex, region of capture, year of capture, and specimen identifier. Lowercase English letters indicate significant differences between months, as measured in the previous panel. **C**. Reproductive activity in *Pn. corruscus* also peaks in the spring. The bars depict the number of mating pairs observed per week on iNaturalist for this species in Connecticut, New Jersey, New York, Pennsylvania, and Vermont, in 2021, 2022, and 2023. The photograph of a type 1 copulation event (Wing, 1984) was taken in New York state in 2021 by Benoît Béchade. **D**. For unidentified species from the *Photuris versicolor* group, the highest bacterial densities are found in specimens from the least urbanized habitats. A GLMM was used to test whether bacterial titer is influenced by habitat type in these species, when controlling for sex, latitude, year and time of capture, day of year, specimen identifier, and whether the specimen was dissected or not. Lowercase English letters indicate significant differences across habitat types. **E**. Bacterial absolute abundance is higher in females than in males of *Pn. pyralis* and *Pn. scintillans*. A GLMM was used to test whether bacterial titer is different between females and males in firefly species (i.e., through the interaction term sex:species), when controlling for the factors as in the previous panel. **F**. Firefly juveniles harbor a higher bacterial diversity than adults, but the two life stages do not differ in their absolute bacterial abundance. GLMMs were used to test whether bacterial titer, bacterial richness, and the bacterial Shannon diversity index are different between juveniles (including larvae and pupae) and adults, using firefly genus as a covariate and controlling for region, day of year, and year of capture. For panels A, D, E, and F, bacterial titer is expressed as the $\log_{10}$ of the number of 16S rRNA gene copies in a sample, measured through qPCR, minus the number of 16S rRNA gene copy in the DNA extraction blank sample from the same batch, and then divided by the total DNA concentration in the sample, which was measured via Qubit. For all panels except C, predicted data (curve for A and B and balls for D, E, and F) with 95% confidence interval (grey shade for A, B, and C, and error bars for D, E, and F) were obtained from GLMMs.

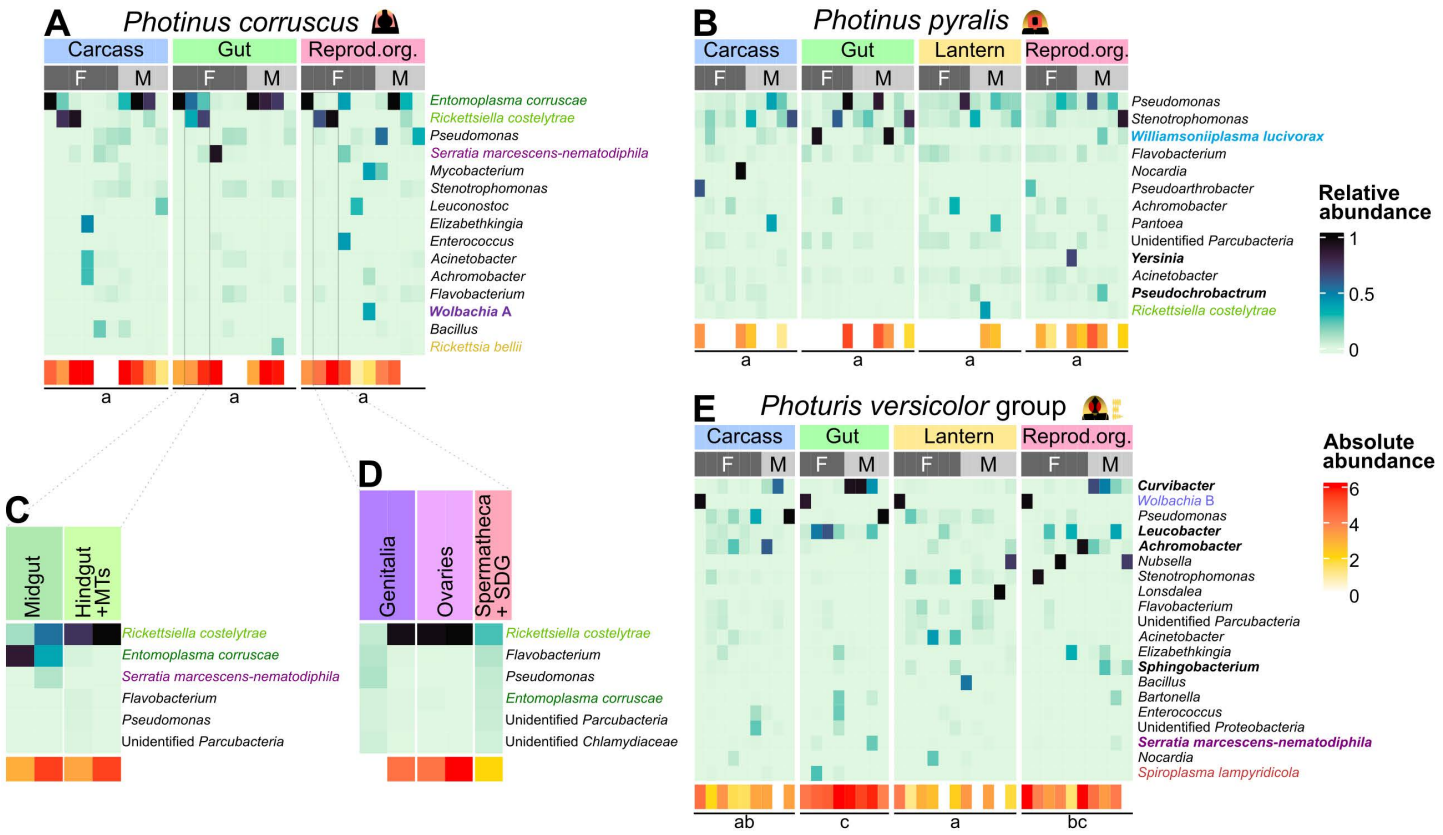

Figure 7: Microbiome changes across tissues in three firefly species. A, **B**, and **E**. Bacterial community composition and abundance across the carcass, gut, reproductive organs ("Reprod. Org."), and lantern in *Photinus corruscus*, *Pn. pyralis*, and unidentified species from the *Photuris versicolor* group. Blue shaded heatmaps show the relative abundance of the most abundant bacterial taxa for each tissue per specimen. The abundance was calculated from non-rarefied, proportional (i.e., compositional) transformations of read depth relative abundance. Bacterial taxa with bold fonts are significantly differentially abundant between at least two tissues in the species, as tested through *MaAsLin2* multiple generalized linear mixed models (GLMMs), using the cumulative sum scaling normalization method, controlling for geographic location and date of sampling, and using the FDR-adjusted $P < 0.05$ as significance threshold. All the differentially abundant bacterial taxa across tissues can be found in our Table S11. Red shaded heatmaps show non-normalized absolute bacterial abundance, expressed as the $\log_{10}$ of the number of 16S rRNA gene copies in a sample, measured through qPCR, minus the number of 16S rRNA gene copy in the dissection blank sample, minus the number of 16S rRNA gene copy in the DNA extraction blank sample from the same batch. A linear mixed model (LMM) was used to compare bacterial titer across tissues in these species, by using the species:tissue interaction term as the fixed effect, controlling for sex, latitude, day of year, year of capture, and temperature. For each firefly species, lowercase English letters indicate significant difference in absolute bacterial titers across tissues, as obtained from emmeans post-hoc tests on the LMM. **C** and **D**. Total absolute bacterial abundance and relative abundance of the six most abundant bacteria in separated gut compartments and reproductive organs of two *Pn. corruscus* female specimens. "MTs": Malpighian tubules and "SDG": spermatophore-digesting gland. More detailed heatmaps and dissection pictures can be found in Figs. S22–S24, and dissection information can be found in Table S2.