Research Article

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 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), whereas deterministic forces involve microbe-microbe interactions (e.g., competition, facilitation) and host/habitat filtering—within-host conditions or microbial traits 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) and evolve adaptations to a host-associated lifestyle (Kwong and Moran 2015), such as reliable transmission modes (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 (Bäumler and Fang 2013; Mazel et al. 2018). Conversely, symbiont communities with lower host specialization, typically including microbes acquired either from the environment or from physical contact with other hosts, 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 symbiosis between microbes and eukaryotes emerge and function as stable associations, it is crucial to identify the importance of each of these ecological processes and their interplay.

42 Fireflies (*Coleoptera*: *Lampyridae*) comprise >2,600 species worldwide (Keller 2025), of
43 which 179 have been described in the US and Canada (Fallon et al. 2021; FireflyAtlas.org 2025).
44 Seminal work using culture-based assays has shown that these insects commonly harbor bacteria
45 from the *Mollicutes* class (Hackett et al. 1992; Stevens et al. 1997; Tully et al. 1989; 1994;
46 Williamson et al. 1990), suggesting a role of deterministic processes in developing stable
47 microbe-host associations. This discovery was recently supported through sequencing-based
48 studies which hinted at possible species-specific associations between a few firefly species and
49 certain bacterial genera (Fallon et al. 2018; Green et al. 2021). Other microbes have also been
50 sampled from fireflies, including common insect endosymbionts (i.e., symbionts residing inside
51 the body or cells of another organism) (Jeong et al. 2009; Jeyaprakash and Hoy 2000) and likely
52 environment-derived bacteria (Green et al. 2021; Zhao et al. 2023), suggesting a role of
53 stochastic processes. However, a more extensive characterization of the firefly microbiome
54 across degrees of relatedness, species lifestyles, and environmental factors is required to better
55 comprehend the nature of firefly-bacteria associations and identify the contribution of ecological
56 forces to their microbiome assembly.
57 While the biology of a large proportion of firefly species is uncharacterized, these insects
58 exhibit diverse life histories and ecologies (e.g., Faust 2017; Lloyd 2018) that can affect, or be
59 affected by, associated microbial communities in deterministic ways. First, as holometabolous
60 insects that include larval, pupal, and adult stages, the ecology and physiology of individual
61 fireflies change drastically throughout their lifetime, potentially resulting in different
62 microbiomes at each developmental stage. In addition, while the adult lifespan is only a few
63 weeks in most firefly species, it is much longer in some, such as the winter firefly (*Photinus*
64 *corruscus*) which can overwinter as adults for over 10 months (Deyrup et al. 2017; Rooney and

65 Lewis 2000). Age may be a key factor impacting resistance to bacterial pathogen infections in
66 fireflies (Lower et al. 2023), but little is known about its influence on the firefly microbiome in
67 the wild. Second, while almost all firefly larvae are predators of soft invertebrates like snails
68 (Buschman 1984a; 1984b; 1988; Fu and Benno Meyer-Rochow 2013; Hess 1920; Vaz et al.
69 2021; 2020; Yang et al. 2024), adult diets are diverse across species and may impose drastically
70 divergent physiochemical conditions upon microbial gut colonizers. For instance, members of
71 the *Photuris versicolor* species group prey on other non-photurine fireflies (Lewis et al. 2011;
72 Lloyd 1965), whereas adult *Pn. corruscus* consume sap and nectar (Rooney and Lewis 2000).
73 Most other firefly species, including *Lucidota* spp., *Pyractomena* spp., and most *Photinus* spp.,
74 are assumed not to eat as adults in the wild (Lloyd 2005; Wing 1989; but see Faust 2017; Faust
75 and Faust 2014; Lloyd 1998; Othman et al. 2018). Third, while bioluminescence is used by all
76 firefly species at the larval stage as a predator avoidance mechanism (Oba et al. 2020; Powell et
77 al. 2022), some species have lost the ability to emit light as adults over evolutionary time (Martin
78 et al. 2017; Stanger-Hall and Lloyd 2015; Stanger-Hall et al. 2007; Zaragoza-Caballero et al.
79 2023). Bacterial symbionts are often implicated in bioluminescence of invertebrates (Cassells et
80 al. 2024; Jones and Nishiguchi 2004), but nothing is known about microbial involvement in
81 firefly bioluminescence, nor about how microbiomes change between hosts that emit light as
82 adult and those that do not. With all these specificities, fireflies provide a rich system to
83 understand interactions between the microbiota and host ecologies, yet little is known about
84 specialization of microbes to these insects.

85 Here, focusing on field-collected fireflies from 12 common species and species groups
86 from the Northeastern United States (Figure 1A and 1B; Figure S1; Table S1), we investigate the
87 degree of host-symbiont specificity and the contribution of diverse factors in structuring firefly

88 microbiomes, such as host species relatedness, diet, bioluminescence, season, geography,
89 urbanization, sex, life stage, and intraindividual tissue localization. Through a combination of
90 quantitative (q)PCR and amplicon sequencing of the bacterial 16S rRNA gene (Figure S2), we
91 tested the hypothesis that most bacteria composing firefly microbiomes are non-specific and
92 these communities are mainly shaped by stochastic forces, except for host species-restricted
93 bacterial strains from the *Mollicutes* which establish through deterministic processes. We predict
94 that while the relative abundance of most bacteria and total bacterial titers vary in fireflies, most
95 firefly species host their own mollicute taxon. This study, focusing on a beetle group that
96 includes differing degrees of symbiont specialization, provides an important contrast to other
97 beetles and paves the way for future research on bacterial transmission modes and influence of
98 microbial symbionts on firefly biology.
99

100 ## Results

101 **1. The microbiome of fireflies is mostly structured by stochastic processes, except when**
102 **bacteria from the *Mollicutes* are included.** To assess the relative importance of deterministic
103 *vs.* stochastic factors in structuring firefly bacterial communities, we calculated and compared
104 the normalized stochastic ratio (NST; Ning et al. 2019) across species that had more than two
105 specimens in our dataset. We found that the microbiome of all nine firefly species tested had an
106 NST index below 0.5 (average = 0.27, SD = 0.10, median = 0.26; Figure 2; Table S2), indicating
107 that their microbiome is structured by deterministic processes. To infer which bacterial group
108 may be responsible for deterministic community assembly, we next removed all the *Mollicutes*
109 from our datasets, and found that the NST significantly increased for five out of nine firefly
110 species (Wilcoxon signed-rank test for these five species: $W < 2.3 \times 10^4$, $P < 0.05$), reaching

index values above 0.5 (for these five species: average = 0.62, SD = 0.09, median = 0.58). This suggests that for several firefly species, while stochastic factors are involved in structuring most of the bacterial community, deterministic processes shape the relative abundance of mollicutes, influencing the whole microbiome. Interestingly, this was not the case for members of the *Photinus marginellus* group, for which removing bacteria known as endosymbionts (i.e., symbionts residing inside the body or cells of another organism) of arthropods (i.e., *Candidatus* Hepatincola, *Rickettsia*, *Rickettsiella*, *Wolbachia*) instead significantly increased the NST index, reaching a value of 0.56 (Wilcoxon signed-rank test: $W = 370$, $P < 0.001$). This indicates that for this firefly species group, deterministic processes shape the abundance of endosymbionts, not mollicutes.

2. The microbiome of fireflies is phylogenetically structured.

2.a. Firefly genera differ in their bacterial density and diversity. Using qPCR, we found that *Photuris* fireflies had significantly higher bacterial densities than *Photinus* fireflies (emmeans: z-ratio = 4.64, $P < 0.0001$; Table S3), despite high intraspecific variability (Figure 1C; Figure S3). The *Pyractomena* and *Lucidota* genera were much less represented in our dataset and members of these groups appear to have intermediate but variable bacterial levels. When looking within firefly genera, we found that *Pn. corruscus* differed from other *Photinus* species in that specimens from this long-lived adult, day-active species had three orders of magnitude higher bacterial titers, reaching levels close to those of *Pr. lucicrescens*. The difference between *Pn. corruscus* and other *Photinus* species was, however, only significant when comparing bacterial titers of *Pn. corruscus* to *Pn. pyralis* (emmeans: z-ratio = 3.67, $P < 0.05$). This indicates that, with the exception of *Pn. corruscus*, members of the *Photinus* genus tend to be infected

with low to extremely low bacterial densities in the wild, while *Photuris* fireflies have moderately high to very high bacterial titers.

In terms of bacterial richness, despite having relatively low bacterial densities, fireflies from the *Photinus* genus harbored significantly more bacterial ASVs than *Photuris* fireflies (emmeans: z-ratio = 4.38, $P < 0.001$; Figure 1D; Figure S4; Table S3 and S4), suggesting that fireflies with higher bacterial densities tend to harbor fewer bacterial species.

2.b. Bacterial composition differs across firefly subfamilies, genera, and species. Following rarefying and aggregation at the bacterial taxon level, we tested differences across microbiome compositions. The microbiome of Northeastern fireflies differed between the *Lampyrinae* and *Photurinae* subfamilies (PERMANOVA: $R^2 = 0.07$, $F_{1,264} = 22.95$, $P < 0.001$; Figure 3A; Table S3) and across genera (nested within subfamilies, PERMANOVA: $R^2 = 0.02$, $F_{2,264} = 3.87$, $P < 0.001$; Figure 3B). Despite high intraspecific variability, bacterial composition also differed significantly across firefly species (nested within genera, within subfamilies, PERMANOVA: $R^2 = 0.09$, $F_{7,264} = 4.18$, $P < 0.001$; Figure 3C–3F). To assess whether firefly microbiomes were more divergent in species that were less genetically related, we examined how bacterial community composition varied with degree of host relatedness. We found a strong positive correlation between pairwise microbiome dissimilarities and host genetic divergence, with closely related firefly species having more similar microbiome composition and abundance (partial Mantel test controlling for geographic distance: $r = 0.37$, $P < 0.001$; Figure S5), further indicating phylogenetically structured microbiomes in fireflies.

2.c. Firefly species that include individuals with bigger size tend to harbor higher bacterial densities. We investigated whether three key characteristics of firefly species—the use of bioluminescence as a mating signal by adults, adult diet, and adult body size—influenced

157 firefly microbiomes when taking into account fireflies' degree of relatedness. Through
158 phylogenetic generalized least square models, we found that bacterial density, richness, and
159 composition did not significantly change between bioluminescent and non-bioluminescent
160 fireflies and between species eating and not eating at the adult stage (Figure S6; Supplementary
161 Text 1; Table S3). We also found that bigger firefly species tend to harbor a higher bacterial
162 density and a lower bacterial richness than smaller species (Figure S7; Supplementary Text 1).
163 These results show that firefly microbiomes do not appear to be influenced by mating signals and
164 adult diet, but that species' body size may contribute.
165
166 **3. Firefly microbiomes are often dominated by a few bacterial taxa with signs of species-**
167 **specific associations.** The most abundant bacterial order associated with all fireflies in our
168 amplicon sequencing dataset was *Entomoplasmatales* (class *Mollicutes*), representing 27.3% of
169 all rarefied bacterial reads from fireflies. Highly represented bacterial orders in fireflies also
170 included *Burkholderiales* (9.5% of all rarefied reads), *Xanthomonadales* (8.6%),
171 *Pseudomonadales* (8.3%), *Enterobacterales* (7.7%), and *Rickettsiales* (7.0%). Compared to non-
172 lampyrid elateroid beetles, only a few of the abundant firefly-associated bacterial taxa were
173 similar (Figure S8; Supplementary Text 1).
174 **3.a. Fireflies harbor a diversity of mollicute taxa with prevalent and abundant**
175 **strains and a few endosymbionts.** The fireflies we collected harbored strains from almost all
176 the mollicute taxa that have been sampled from fireflies in the past (seven out of eight; Figure 4;
177 Figure S9; Supplementary Text 1). Both *Spiroplasma ixodetis* and *Williamsoniiplasma lucivorax*
178 species were represented with a high strain diversity in our firefly specimens, with at least 20 and
179 30 ASVs each, respectively. Despite this train diversity, within several mollicute genera and

species, one or two ASVs could be very abundant and prevalent in fireflies, some of which have only been sampled from firefly bodies to date and thus appear endemic to fireflies. While the high abundance of mollicutes associated with fireflies is unusual for an insect microbiome, we note that several prevalent and abundant bacteria in these insects are known to be endosymbionts in other arthropods (Figure 5A; Figure S10). These include bacteria from the *Rickettsia*, *Rickettsiella*, *Wolbachia*, and occasionally *Serratia* and *Spiroplasma* genera (Russell et al. 2013; Werren et al. 2008).

3.b. *Stenotrophomonas* and *Williamsoniiplasma* often dominate lampyrine microbiomes. Following filtering of bacterial taxa with low abundance (keeping only taxa with rarefied abundance > 20,000 reads in one or more samples) in our amplicon sequencing dataset, we found that the *Stenotrophomonas* (*Xanthomonadales*) and *Williamsoniiplasma* (*Mollicutes*) genera were characteristic members of the microbiome of fireflies from the *Lampyrinae* subfamily (Figure 5B), each detected in more than 98% of lampyrine fireflies (colonizing > 57% of lampyrine specimens with more than 1% relative abundance). Compared to fireflies from the *Photurinae* subfamily, lampyrine fireflies were significantly more associated with bacteria from these two genera (MaAsLin2: coef > 3.30, FDR *P*-adj < 0.0001; Table S5). While *Stenotrophomonas* could also be found in *Photuris* fireflies, albeit typically at low abundance, members of the *Williamsoniiplasma* genus were very rare in *Photuris* species (Figure 5A and 5B; Figure S11 and S12), making this bacterial genus nearly endemic to the lampyrine microbiome.

Three *Williamsoniiplasma* taxa were commonly detected in *Photinus* and *Pyractomena* fireflies with differing levels of endemicity (Figure 5A; Figure S11). *Wi. lucivorax* was only found in *Pn. pyralis*, as the most dominant and prevalent bacterium after *Stenotrophomonas*, infecting over 69% of individuals with more than 1% relative abundance, and being significantly

203 more abundant in this firefly species than in any other (MaAsLin2: coef > 4.50, FDR *P*-adj <
204 0.0001; Figure S11; Table S5). In contrast, *Wi. luminosum* was detected in both *Pn. pyralis* and
205 *Pn. scintillans*, as well as in two individuals from the *Pn. marginellus* group (Figure 5A; Figure
206 S11), thus appearing less host-restricted than *Wi. lucivorax*. Yet, *Wi. luminosum* was
207 significantly associated with *Pn. scintillans* (*vs.* all firefly species but *Pn. marginellus* group:
208 MaAsLin2: coef > 1.55, FDR *P*-adj < 0.05; Figure S11), being found in all *Pn. scintillans*
209 specimens and 64% of *Pn. scintillans* specimens with 1% relative abundance threshold (Figure
210 5A). Another species from the *Williamsoniiplasma* genus, *Wi. somnilux*, was significantly more
211 abundant in *Pyractomena borealis* than in other firefly genera (MaAsLin2: coef > 8.04, FDR *P*-
212 adj < 0.0001; Table S5) and showed endemicity patterns similar to those of *Wi. lucivorax* (Figure
213 5A; Figure S11). When using the 1% relative abundance threshold, *Wi. somnilux* was present in
214 five out of eight specimens (62%) from this firefly species, including both adults and juveniles,
215 and was the most abundant bacterial taxon (Figure 5A). We also found that *Pn. corruscus*
216 fireflies were significantly associated with another member of the *Mollicutes*, the *En. corruscae*
217 taxon (MaAsLin2: coef > 4.30, P-adj < 0.001; Figure 5A; Figure S11), being the most abundant
218 bacterial taxon in all *Pn. corruscus* fireflies, found at more than 1% relative abundance in nine
219 out of 16 individuals (56%), and showing patterns of endemicity as well.

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

224 **3.c. Photurine fireflies commonly harbor a variety of bacteria, including a few**
225 **mollicutes.** Contrasting with the lampyrines, members of the *Photurinae* were significantly more

226 associated with abundant bacteria (filtered taxa with > 20,000 reads in one or more samples)
227 from the *Achromobacter* (*Burkholderiales*), *Ca.* Hepatincola (*Rickettsiales*), *Curvibacter*
228 (*Burkholderiales*), *Elizabethkingia* (*Flavobacteriales*), *Enterococcus* (*Lactobacillales*), and
229 *Spiroplasma* (*Mollicutes*) genera (MaAsLin2: coef > 1.87, FDR *P*-adj < 0.0001; Figure 5B).
230 Despite a lower relative abundance, *Leucobacter* bacteria also appeared significantly associated
231 with photurine fireflies (MaAsLin2: coef = 2.97, FDR *P*-adj < 0.0001; Figure S11 and S12;
232 Table S5). Among the spiroplasmas, two taxa were significantly more abundant in photurine
233 fireflies: *Sp. ixodetis* and *Sp. lampyridicola* (found in 14% and 7% of *Photuris* specimens at 1%
234 relative abundance threshold, respectively) (MaAsLin2: coef > 3.25, FDR *P*-adj < 0.0001).
235 Another mollicute species, *Tullyiplasma photuris*, was abundant in photurine fireflies, though at
236 lower degrees (MaAsLin2: coef = 0.69, FDR *P*-adj < 0.05; Figure 5A; Figure S11), found in
237 13% of *Photuris* specimens at 1% relative abundance threshold.
238 **3.d. Bacteria positively and negatively co-occur in fireflies.** From our co-occurrence
239 analysis, we found that some bacteria, including endosymbionts (e.g., *Rickettsia bellii*,
240 *Wolbachia*) and potential pathogens (e.g., *Serratia marcescens-nematodiphila*), were more likely
241 to be found on their own in fireflies (Figure S13). We also found some positive co-occurrence
242 patterns within firefly species, including *Pseudomonas*, *Stenotrophomonas*, and *Rl. costelytrae* in
243 both *Pn. marginellus* group and *Pn. pyralis*, and *Achromobacter*, *Curvibacter*, and
244 *Elizabethkingia* in *Photuris* fireflies. This suggests that some bacteria are more likely to occur
245 alone whereas others may be part of a consortium in fireflies.
246
247 **4. Within-species variation can be partially explained by the region, season, environment,**
248 **sex, and developmental stage.**

4.a. The abundance of some, but not all, bacteria vary across geographic locations. To assess whether geography was a main factor influencing changes in firefly microbiomes, we first used partial Mantel tests on microbiome dissimilarity matrices and geographic distance, while taking into account species' phylogenetic distances. Through this test, we found a weak positive correlation between pairwise microbiome dissimilarities based on relative bacterial abundance and geographic distance (partial Mantel test controlling for genetic distances: $r = 0.05$, $P < 0.05$; Table S3), indicating that regardless of host species identity, fireflies separated by longer spatial distances tend to harbor more dissimilar microbial communities. Because of substantial intra-specific variation in bacterial density and community composition, we next investigated within species microbiome variation for species that were collected in multiple regions.

At the level of the whole bacterial community, *Pn. corruscus* microbiomes did not appear to differ across regions (PERMANOVA: $R^2 = 0.40$, $F_{5,10} = 1.33$, $P = 0.11$; Figure S14; Table S3). Nevertheless, and despite a low sample size ($n = 16$) and potential confounding factors (albeit being controlled as random factors in our GLMMs and MaAsLin2 models), we found regional differences in terms of absolute bacterial abundance (Supplementary Text 1), and at the level of individual bacteria (Figure 6A; Table S6). *En. corruscae*, which was a characteristic bacterial taxon of *Pn. corruscus*, may have preferentially been associated with specimens from northern latitudes, since it was less abundant in specimens collected in southwestern Connecticut (swCT) and South New Jersey compared to specimens collected in northwestern CT (nwCT), southeastern New York (seNY), and southwestern Vermont (swVT) (MaAsLin2: coef > 4.57, FDR P-adj < 0.01).

271 *Pn. pyralis* microbiomes showed significant variation in composition across regions
272 (PERMANOVA: $R^2 = 0.13$, $F_{5,106} = 3.15$, $P < 0.001$; Figure S14; Table S3), despite relatively
273 stable absolute bacterial abundances (emmeans: z-ratio < 1.76, $P > 0.07$). The most divergent
274 microbiomes were from *Pn. pyralis* specimens collected in central Pennsylvania (cPA) (pairwise
275 adonis: $R^2 > 0.08$, $F > 2.99$, FDR P-adj. < 0.05; Figure 6B). Likely contributing to the singularity
276 of cPA *Pn. pyralis* microbiomes, *Wi. luminosum* was mostly found in individuals collected in
277 this region (MaAsLin2: coef > 3.64, FDR P-adj < 0.05; Table S6), where its prevalence at 1%
278 relative abundance threshold was 68% (64% for *Wi. lucivorax* in these specimens) *vs.* 4% in *Pn.*
279 *pyralis* from all other regions (71% for *Wi. lucivorax* in these other specimens). For *Wi.*
280 *lucivorax*, relative abundance was high in specimens collected in several states. *Pn. pyralis*
281 specimens from western Virginia also had a divergent microbiome compared to specimens from
282 other regions, with compositions differing from those of cPA and southeastern PA specimens
283 (pairwise adonis: $R^2 = 0.03$, $F = 2.51$, FDR P-adj. < 0.05), and unusually dominated by bacteria
284 from the *Mycoplasma*-related group (Figure 6B). For both *Pn. marginellus* group and *Pn.*
285 *scintillans*, we found limited evidence of regional specificities in the microbiome
286 (PERMANOVA: $R^2 < 0.17$, $F < 1.67$, $P > 0.11$; Figure 6B; Figure S14), although total bacterial
287 density was higher in *Pn. scintillans* collected in cPA compared to sePA (emmeans: z-ratio =
288 2.03, $P < 0.05$).
289 Regarding the *Photuris* genus, we found strong microbiome differences across regions
290 for the unidentified species of the *Pr. versicolor* group (PERMANOVA: $R^2 = 0.13$, $F_{4,51} = 1.83$,
291 $P < 0.001$; Figure S14; Table S3), but not for *Pr. hebes* (PERMANOVA: $R^2 = 0.11$, $F_{2,19} = 1.18$,
292 $P = 0.24$). Members of the bacterial taxon *Sp. ixodetis* were common and abundant in specimens
293 collected in cPA, but were very rare in specimens from other regions (MaAsLin2: coef > 5.58,

294 FDR *P*-adj < 0.001; Table S6). We also identified regional specificities among other common
295 bacterial associates of *Photuris* fireflies, with *T. photuris* being more abundant in northeastern
296 NY (neNY) and seNY compared to sePA for *Pr. hebes* (MaAsLin2: coef > 5.88, FDR *P*-adj <
297 0.0001), and neNY compared to sePA in unidentified species from the *Pr. versicolor* group
298 (MaAsLin2: coef > 3.53, FDR *P*-adj < 0.05).

299 **4.b. Seasonal, but not annual, factors influence within-species microbiome variation.**
300 We tested whether seasonality, which is linked to firefly adult age, influenced firefly
301 microbiome. When focusing on all species in our dataset, we found that bacterial density varied
302 throughout day of year (GLMM Wald test: $X^2 = 124.38$, $P < 0.0001$; Table S3), but this appeared
303 to be the case mostly for some firefly species. Several species, including *Pn. scintillans*,
304 unidentified *Pr. versicolor* group, and *Py. borealis*, tended to have higher bacterial titers later in
305 the season (emmeans: *z*-ratio > 2.63, $P < 0.05$). We also found that bacterial composition varied
306 throughout the year and across species (PERMANOVA with species and regions as covariates,
307 focusing on the interaction between day of year and species: $R^2 = 0.05$, $F_{14,186} = 1.20$, $P < 0.05$).
308 We next focused our analyses only on *Pn. corruscus* specimens given their long adult lifespan
309 (up to 10 months *vs.* less than three months usually for other species) and since we collected this
310 species throughout the year.

311 *Pn. corruscus* specimens collected in the months of April, May, and June had higher
312 bacterial titers than those collected in November (emmeans: *z*-ratio > 3.55, $P < 0.01$; Figure 7A;
313 Table S3). When looking at individual bacterial taxa, we found that specimens collected in May
314 and April were typically associated with high to very high relative abundance of *En. corruscae*,
315 but this bacterial taxon was barely detectable in *Pn. corruscus* specimens collected in February
316 and November (April and May *vs.* February and November, MaAsLin2: coef > 4.89, FDR *P*-adj

317 < 0.01; Figure 7B; Table S7). Supporting these results across months, we found a strong
318 association between the collection day of year used as a quadratic continuous predictor in our
319 LMMs and both total bacterial titers (GLMM Wald tests: $X^2 > 35.81$, $P < 0.0001$), and
320 abundance of *En. corruscae* (LMM Wald tests: $X^2 > 18.47$, $P < 0.0001$), in *Pn. corruscus*, with a
321 peak in late May (Figure 7A and 7B). Thanks to citizen science data collected from iNaturalist
322 (iNaturalist community 2025), we found that during the years and in the states of collection, the
323 reproductive activity of *Pn. corruscus* spanned from early April to mid/late June, peaking during
324 the month of May (Figure 7C). This corresponds to the reproductive seasons observed for this
325 species in other states and years (Faust 2012; Rooney and Lewis 2000; Smedley et al. 2017), and
326 implies a potential link between *En. corruscae* abundance and either its host reproduction or
327 seasonal increased food intake (Rooney and Lewis 2000).

328 To assess microbiome turnover across years, we collected *Pn. pyralis* specimens in the
329 same location, at approximately the same time of year, for three consecutive years. We found
330 that the overall microbiome composition of *Pn. pyralis* changed only marginally over the years
331 (PERMANOVA: $R^2 = 0.06$, $F_{2,53} = 1.76$, $P = 0.051$; Table S3). However, at the level of
332 individual bacterial taxa, while the abundance of *Wi. lucivorax* remained stable from 2021 to
333 2023, that of *T. photuris* and *Wi. luminosum* increased and that of *Pseudoclavibacter* and
334 *Tumebacillus* dropped (MaAsLin2: coef < -1.96, FDR *P*-adj < 0.05; Figure S15; Table S7). This
335 shows that the overall composition of bacteria associated with fireflies may not drastically
336 change over the years, although the abundance of a few bacteria may fluctuate.

337 **4.c. The microbiome of *Photuris versicolor* group fireflies changes across habitat**
338 **types.** The interaction between species and habitat types (with different urbanization levels)
339 significantly affected both bacterial composition and density (PERMANOVA with species and

340 regions as covariates: $R^2 = 0.05$, $F_{14,235} = 1.30$, $P < 0.001$; GLMM Wald test: $X^2 = 143.6$, $P <$
341 0.0001; Table S3), indicating that different species' microbiomes vary in their response to
342 divergent habitat types. In unidentified species from the *Pr. versicolor* group, we found that
343 absolute bacterial abundance was significantly lower for specimens collected in the city than in
344 the forest or in a mix of forest and meadow (emmeans: z-ratio > 3.17, $P < 0.05$; Figure 7D).
345 Through MaAsLin2 multiple GLMMs controlling for potential confounding factors, we found
346 that *Rickettsiella costelytrae* may have been a key influence on bacterial titers over the
347 urbanization gradient since it was more abundant in specimens from the forest than in the city
348 (MaAsLin2: coef = 5.72, FDR P-adj < 0.01; Figure S16; Table S7). These results show that the
349 type of habitat used by *Photuris* fireflies may influence the abundance and composition of their
350 associated bacteria.

351 **4.d. *Photinus* females harbor a denser bacterial community than males.** Using qPCR,
352 we found that females of both *Pn. pyralis* and *Pn. scintillans* had a significantly higher bacterial
353 titer than their male conspecifics (emmeans: z-ratio > 2.03, $P < 0.05$; Figure 7E; Table S3). In
354 contrast, in *Photinus* species, the Evar index of evenness was higher for males compared to
355 females (emmeans: z-ratio = 3.75, $P < 0.01$; Figure S17), suggesting that bacterial strains in male
356 microbiomes were represented in more equal proportions, while female microbiomes tended to
357 be dominated by fewer bacteria. Nevertheless, firefly microbiome composition did not
358 significantly differ between sexes, with the only exception of *Pr. hebes* (pairwise adonis: $R^2 =$
359 0.09, $F = 1.97$, FDR P-adj < 0.05).

360 **4.e. Juveniles harbor a more diverse bacterial community than adults.** We collected
361 a few larvae from three different firefly genera, and pupae from the *Pyractomena* genus. When
362 comparing adults to juveniles (i.e., including both larvae and pupae) from all firefly species,

363 while we found no difference in terms of absolute bacterial abundance (GLMM Wald test: X^2 =
364 1.08, P = 0.30; Figure 7F; Table S3), there was a significant difference in their microbiome
365 composition (PERMANOVA with life stage nested within species within genus within
366 subfamily: R^2 = 0.01, $F_{3,261}$ = 1.35, P < 0.05), a higher bacterial richness (GLMM Wald test on
367 number of ASVs with firefly genus used as a covariate: X^2 = 4.99, P < 0.05), and a higher
368 bacterial diversity (GLMM Wald test on Shannon diversity with firefly genus used as a
369 covariate: X^2 = 7.40, P < 0.001) in juveniles compared to adults. These results appear to be
370 driven by the difference between larvae and adults (Figure S18; Supplementary Text 1).

371 The single larva from the *Photinus* genus, identified through DNA barcoding as from the
372 *Pn. consanguineus* group (Figure S1), did not appear to have a different bacterial composition
373 compared to adults, and did not harbor any *Williamsoniiplasma* spp. (Figure S19). In contrast,
374 *Pr. versicolor* group larvae had a different bacterial composition compared to adults
375 (PERMANOVA: R^2 = 0.03, $F_{1,84}$ = 2.22, P < 0.01; Table S3), including a significantly higher
376 abundance of unidentified *Rhizobiales* (MaAsLin2: coef = 8.92, FDR P-adj < 0.05; Table S7).
377 Notably, one larva specimen from this species group had a higher bacterial titer compared to the
378 two other larvae and harbored *Sp. ixodetis* with high relative abundance. All three *Photuris*
379 larvae analyzed hosted *Leucobacter* in high relative abundance, which was also commonly found
380 in adults (Figure S11 and S12).

381 Juveniles from the *Py. borealis* species did not have a significantly different bacterial
382 community as a whole when compared to adult males from the same species (PERMANOVA: R^2
383 = 0.25, $F_{1,6}$ = 1.99, P = 0.11; Figure S19; Table S3). Nevertheless, and despite our low sample
384 size, significant differences in the abundance of single bacteria across larvae, pupae, and adult
385 males could be noted. The mollicute *Wi. somnilux*, which was commonly detected in *Py. borealis*

386 fireflies, associated with adults and larvae at similar abundances (MaAsLin2: coef = 0.14, FDR
387 *P*-adj = 1; Figure S20; Table S7), but was present in lower abundance in pupae (MaAsLin2: coef
388 = 7.92, FDR *P*-adj < 0.01). Other bacterial genera, including *Rhodococcus*, had a significantly
389 higher abundance in *Pyractomena* juveniles compared with adult males (MaAsLin2: coef > 1.99,
390 FDR *P*-adj < 0.05; Figure S19). These results indicate that juveniles harbor a more diverse
391 microbiome, and some of the most prevalent and abundant adult-associated bacteria are also
392 abundant in larvae and detected in pupae.
393
394 **5. The tissue localization of bacteria varies across firefly species.**
395 We investigated preferential colonization of bacteria in specific tissues of three firefly species
396 and species groups through dissections and tissue separations (Table S8). We found a significant
397 difference in overall bacterial composition across firefly tissues, but only for unidentified species
398 from the *Pr. versicolor* group, between their lantern and gut tissues (pairwise adonis: $R^2 = 0.12$,
399 $F = 2.36$, FDR *P*-adj < 0.01; Figure S21; Table S3), indicative of major tissue differences only in
400 *Photuris* fireflies.
401 **5.a. *Pn. corruscus* tissues do not differ in bacterial titer but have a few differentially**
402 **abundant bacterial taxa.** Bacterial densities were relatively high throughout the body of *Pn.*
403 *corruscus* (Figure 8A). In terms of composition, *En. corruscae* commonly colonized all the main
404 tissues of both males and females, suggesting that most *Pn. corruscus* tissues are colonized by
405 bacteria in high abundance, including prevalent mollicutes.
406 Two females had their gut segments and reproductive organs separated through
407 dissection. The mollicute *En. corruscae* was not found in ovaries of these females, but was
408 present in other reproductive organs, and was very abundant in the midgut (Figure 8A, 8C, and

409 8D; Figure S22). In contrast, *Rl. costelytrae* was very abundant in *Pn. corruscus*' carcass,
410 hindgut, and ovaries and was also detected in the genitalia, spermatheca and spermatophore-
411 digesting gland, and midgut of these two individuals, matching infection patterns typical of
412 symbionts residing inside host tissues (i.e., endosymbionts; Perlmutter and Bordenstein 2020).
413 Mollicutes and endosymbionts may thus slightly differ in their tissue localization in *Pn.*
414 *corruscus*, *En. corruscae* being mainly found in the midgut but also infecting reproductive
415 organs, and endosymbionts such as *Rickettsiella* colonizing most tissues, including ovaries.

416 **5.b. Bacteria variably colonize the tissues of *Pn. pyralis* but with a potential primary**
417 **gut localization of *Williamsoniiplasma*.** While there were no significant differences in bacterial
418 titers across *Pn. pyralis* dissected tissues (emmeans: t-ratio < 1.93, $P > 0.22$; Figure 8B), the gut
419 and reproductive organs could occasionally be infected with high titers. When present, *Wi.*
420 *lucivorax* was found to be common and relatively abundant in the gut, and while it was
421 sometimes detected in the carcass and reproductive organs of males, it appeared virtually absent
422 from the lantern (MaAsLin2: coef > 3.79, FDR P-adj < 0.05; Figure 8B; Figure S23; Table S9).
423 Despite being much less abundant overall than its sister species, the abundance of *Wi. luminosum*
424 was also reduced in the lantern compared to the gut (MaAsLin2: coef = 2.40, FDR P-adj <
425 0.001). This suggests that, as for *Pn. corruscus*, mollicutes may have a primary gut localization
426 in *Pn. pyralis*, but with lower bacterial titers overall in this firefly species.

427 **5.c. Specimens from the *Photuris versicolor* group harbor high density gut bacteria.**
428 While bacterial density in unidentified species from the *Pr. versicolor* group whole body was
429 relatively high (Figure 1C; Figure S3), it peaked in the gut, especially compared to the lantern
430 and carcass (emmeans: t-ratio > 2.85, $P < 0.05$; Figure 8E; Figure S24; Table S3). All
431 individuals of these species had a consistently high bacterial titer in the gut, regardless of sex,

432 collection date, and location. Reproductive organs also had significantly higher bacterial titers
433 than the lantern (emmeans: t-ratio = 2.71, $P < 0.05$).
434 When separating the unidentified *Pr. versicolor* group specimens by sex, we found that
435 bacteria from the *Curvibacter* genus dominated the microbiome of male guts and reproductive
436 organs compared to female guts and other male tissues (male gut vs. all other tissues except male
437 reproductive organs: MaAsLin2: coef > 4.19, FDR P-adj < 0.05; Figure 8E; Table S9).
438 Otherwise, the gut of both females and males included a high abundance of *Leucobacter* in the
439 gut and reproductive organs compared to the carcass and the lantern (MaAsLin2: coef > 2.93,
440 FDR P-adj < 0.05). Notably, one female harbored *Wolbachia* from the supergroup B in all its
441 tissues and another one had a high abundance of *Sp. lampyridicola* in its gut (Figure 8E).
442 *Photuris* fireflies thus harbor high density gut bacteria with compositions that seem to differ
443 between males and females.
444

445 **Discussion**

446 We studied the factors shaping firefly microbiomes through a comprehensive characterization of
447 bacterial communities in several Northeastern US firefly species. Echoing previous research
448 (Green et al. 2021; Hackett et al. 1992), we detected a high variation in bacterial composition
449 and abundance within firefly species (Figure 1C and 5A; Figure S3, S12, and S19).
450 However, we also reveal that the bacterial community of most firefly species is unique (Figure
451 3C–3F), implying that each host species preferentially associates with its own set of bacteria.
452 This high species-specificity combined with intraspecific variability could be explained by the
453 interplay between stochastic and deterministic mechanisms. This was supported by our analysis
454 of normalized stochasticity ratios (NST), showing that either mollicutes or endosymbionts (or

455 both in some *Photuris* species) were acquired by fireflies through deterministic factors, but when
456 these symbionts were not included in the microbiome the rest of these firefly-associated bacterial
457 communities were mainly structured through stochastic forces (Figure 2). These results highlight
458 the importance of partitioning microbiome analyses based on microbial incidence (e.g.,
459 *Mollicutes*) and known functional groups (e.g., endosymbionts), as different ecological processes
460 dictate their acquisition and maintenance by hosts.
461

462 **Ecological mechanisms structuring firefly microbiota**

463 Mollicutes and some endosymbionts may be acquired and maintained through deterministic
464 forces, such as habitat/host filtering mechanisms, which include some component of the host
465 physiology favoring the acquisition, transmission, and establishment of only specific microbes
466 (Mazel et al. 2018; Michalik and Szklarzewicz 2025; Ohbayashi et al. 2015; Sachs et al. 2004),
467 and bacteria-driven exclusion or facilitation of the establishment of other bacteria (Alizon et al.
468 2013; Zélé et al. 2018). Other bacteria composing firefly microbiomes may largely be free-living
469 bacteria acquired from the environment, and their acquisition may depend on stochastic
470 mechanisms, such as random exposure events experienced individually by each firefly (Jones et
471 al. 2022; Zhou and Ning 2017). We further discuss these mechanisms and how they contribute to
472 structuring the firefly microbiome below.

473 First, some of our results support the idea that variability in firefly microbiomes is due to
474 stochastic community assembly mechanisms, including the random acquisition of various free-
475 living microbes from the environment, as suggested before (Green et al. 2021). For example, we
476 found that, for several firefly species, the prevalence of individual bacterial taxa changed across
477 specimens collected in different geographic locations (e.g., *Lactococcus* in *Photinus* species;

478 Figure 6A–6B). A similar variation was observed in *Photuris* microbiomes across habitats with
479 different levels of urbanization (Figure 7D; Figure S16). Such intraspecific variability of
480 microbiome composition has been described in a wide range of host groups like butterflies or
481 mammals (Ley et al. 2008; Ravenscraft et al. 2019). Acquisition of several bacteria from
482 environmental pools, which differ in their composition across geographic locations and habitat
483 types (Hosen et al. 2017; Malard et al. 2019; Martiny et al. 2006; Zheng et al. 2024), thus
484 appears probable in fireflies.

485 Second, deterministic habitat filtering mechanisms imposed by host physiological
486 conditions are likely to participate in shaping the establishment of characteristic members of the
487 bacterial communities. Supporting this, we found that, despite being collected at the same
488 locations, times of day, and seasons, sympatric firefly species (Faust 2017; Lloyd 2018; Martin
489 et al. 2024; Stanger-Hall and Lloyd 2015) did not share similar microbiomes, whether they were
490 closely related or not (Figure 3A–3F). As previously pointed out by Hackett et al. (1992), even
491 fireflies from the *Photuris versicolor* group, which consume *Photinus* and *Pyractomena* fireflies,
492 did not harbor any of the *Williamsoniiplasma* bacteria characteristic of their prey (Figure 5A and
493 5B). In addition, several firefly-associated bacterial taxa from the *Mollicutes* may be endemic to
494 fireflies since they have only been found in firefly tissues to date (Figure 4; Figure S8 and S11).
495 These results are indicative of host restriction, suggesting that fireflies are not entirely permeable
496 to the microbes they are exposed to, and their microbiome composition cannot be fully explained
497 by passive colonization of environmental bacteria. Unknown habitat filtering mechanisms within
498 the host are thus likely to contribute to the assembly of firefly microbiomes, as is the case in
499 other organisms (Ohbayashi et al. 2015; Sachs et al. 2004).

500 Vertical transmission can be considered as a special case of deterministic habitat/host
501 filtering mechanism, where only specific symbionts are transmitted from parents to offspring and
502 conserved by the host line throughout generations. Many insects associating with lineage-
503 specific, primary symbionts have been shown to rely on strict vertical transmission to preserve
504 key beneficial functions from the symbiont (Baumann 2005; McCutcheon et al. 2009; Sacchi et
505 al. 2000). Yet, several endosymbionts that are common across arthropods, such as *Rickettsia*,
506 *Rickettsiella*, *Spiroplasma*, and *Wolbachia* practice a mix of vertical and horizontal transmission
507 with no strict host specificity (Gu et al. 2023; Liu et al. 2024; Michalik and Szklarzewicz 2025;
508 Perreau and Moran 2022). Here, we confirmed the endosymbiotic nature of *Rickettsiella* and
509 *Wolbachia* in fireflies, which infected several tissues, including reproductive organs (Figure 8A–
510 8E). While our NST analysis suggests that the acquisition of these endosymbionts was
511 deterministic in *Pn. marginellus* group and unidentified species from the *Pr. versicolor* group
512 (Figure 2), these endosymbionts appeared with a more random distribution in other firefly
513 species (Figure 5A), suggesting a stochastic influence, perhaps conditioned by horizontal jumps
514 across hosts. Although we detected endosymbionts and mollicutes in female reproductive organs
515 (Figure 8A), it is still unclear whether they are vertically transmitted to firefly offspring.
516 Third, bacteria-driven exclusion or facilitation is another deterministic process that could
517 structure firefly-associated bacterial communities. In insect microbiomes, some bacteria have to
518 compete to establish in a host (Itoh et al. 2019), while others need other bacterial species to be
519 present (Carpenter et al. 2021). We found that when they were detected in fireflies, *Rickettsia*
520 and *Wolbachia* were each likely to dominate bacterial communities and rarely co-occurred with
521 other bacteria (Figure S13). This result could be a consequence of potential competitive
522 exclusion driven by these known endosymbionts (Caragata et al. 2013). However, we cannot

523 reject the possibility that these endosymbionts were present at such high densities in firefly
524 tissues that the sequencing depth was not high enough to detect other co-occurring bacteria
525 (Gofton et al. 2015). We note that *Rickettsia bellii* and *Wolbachia* supergroup B usually had a
526 very high absolute bacterial abundance in specimens (Figure S19 and S24), supporting this latter
527 possibility. We also found patterns of positive co-occurrence among bacteria associated with a
528 few firefly species, which could suggest facilitation. Chief among them is the *Pseudomonas*-
529 *Stenotrophomonas* duo found in several *Photinus* fireflies (Figure S13), also known to cooperate
530 during infections of vertebrates (McDaniel et al. 2023; 2020). In *Photuris* fireflies, *Curvibacter*,
531 *Achromobacter*, and *Elizabethkingia* tend to co-occur in the reproductive organs (Figure 8E) and
532 in the gut (Green et al. 2021), suggesting acquisition of this trio either from sexual transmission
533 or from food intake. Future work should investigate whether these bacteria are acquired together
534 by chance (Carpenter et al. 2021), or truly facilitate each other's establishment in firefly hosts
535 (Halliday et al. 2024; Zélé et al. 2018).
536 It thus appears that, as in many animals (Chandler et al. 2011; Furman et al. 2020;
537 Hendrycks et al. 2025; Oliphant et al. 2019), fireflies acquire their microbiomes through a
538 combination of stochastic and deterministic elements. Mechanisms involved likely include the
539 acquisition of environmental bacteria, habitat filtering mechanisms—including potential reliable
540 transmission modes, partner choice processes to select specific bacterial strains, and/or parasitic
541 associations where parasites circumvent barriers to establishment—and possible competition and
542 facilitation among bacterial strains. Future studies should test the contribution of each of these
543 stochastic and deterministic mechanisms in structuring firefly microbiota.
544
545 **Which factors influence the association with bacterial symbionts?**

546 As a group, fireflies offer diverse ecologies across species and throughout their lifetime. We
547 investigated the influence of several firefly traits, such as sex, dietary ecology, and life stage, on
548 the composition and abundance of housed bacteria. Notably, we did not find convincing
549 evidence directly linking adult bioluminescence to bacterial symbiosis, since bioluminescent
550 firefly species had similar bacterial loads and composition as non-bioluminescent ones (Figure
551 S6) and the lantern of two common species housed either low or variable bacterial density with
552 seemingly random bacterial composition, except in the rare occasion of infections with an
553 endosymbiont colonizing all tissues including the lantern (Figure 8B and 8E; Figure S23 and
554 S24).

555 Interspecific variation in the animal microbiome is often influenced by diet (Degregori et
556 al. 2024; Karasov and Douglas 2013). Here, we found that bacterial density and composition
557 changed between fireflies known to eat versus those supposed not to eat as adults (Figure S6).
558 However, since diet and phylogeny are confounded in our study, this result was not well
559 supported when accounting for host phylogenetic relationship, which was responsible for a large
560 portion of microbiome (dis)similarity among firefly species (Figure 3A–3C; Figure S5). Notable
561 microbiome divergences observed between closely related firefly species could, nevertheless, be
562 attributed to species' differing ecology, perhaps including dietary differences. For example, *Pn.*
563 *pyralis* and *Pn. corruscus*, which are two closely related species (Catalan et al. 2022) with
564 overlapping geographic range—the former not seemingly eating as adults and having a short
565 adult lifespan from May to August, and the latter consuming plant sap and nectar and living from
566 November to May as adults—harbored divergent bacterial densities and compositions (Figure 1C
567 and 3F). In-depth dietary analyses should be performed to formally address the contribution of

568 adult diet to microbial abundance and composition in fireflies, while controlling for host
569 relatedness.

570 Our results suggest that some of the intraspecific variation in bacterial abundance in *Pn.*
571 *corruscus* could be explained by seasonal effects. We found that the abundance of the mollicute
572 *Entomoplasma corruscae* associated with these fireflies increased in the spring (Figure 7B),
573 when reproductive activity peaks for this firefly species in the Northeastern US (Figure 7C).
574 Given that this bacterium was sometimes abundant in the midgut, but could also be detected in
575 the reproductive organs and carcass (Figure 8A and 8D; Figure S22), several possible modes of
576 acquisition may promote the proliferation of *En. corruscae* in the spring. First, *Pn. corruscus*
577 fireflies may drastically increase food intake during the reproductive season (Rooney and Lewis
578 2000), hence acquiring large quantities of this bacterium from their food sources, thus implying
579 an environmental acquisition mode. But it is also possible that *En. corruscae* is vertically
580 transmitted from parents to offspring, although this was not supported in a recent study analyzing
581 the bacterial composition of six eggs (Green et al. 2021). Finally, as seen in the association
582 between aphids and three facultative endosymbionts (Moran and Dunbar 2006), *En. corruscae*
583 could potentially be sexually transmitted (i.e., horizontal transmission), and its abundance in
584 adults could be directly proportional to the number of copulation events they experience.

585 As holometabolous insects, fireflies have two dissociated, active life stages with
586 drastically divergent ecologies, translating into distinct periods of exposure to, and interaction
587 with, microbes (Hammer and Moran 2019; Manthey et al. 2023): one at the larval stage, after
588 eggs hatch, and one at the adult stage, following pupation. While the ecology, physiology, and
589 behaviors of adult fireflies have been well documented, earlier developmental stages (e.g., eggs,
590 larvae, pre-pupae, and pupae) remain understudied, largely due to the cryptic lifestyles of these

591 early stages (e.g., soil dwelling) and difficulties to rear them throughout their development (Riley
592 et al. 2021). However, it is crucial to study larval microbiomes, since fireflies spend most of their
593 life in this stage. Here, we found that firefly larvae from the Northeastern US harbored a more
594 diverse bacterial community compared to adults (Figure 7F; Figure S18), with diversity index
595 values and a few bacterial genera similar to those from a study on the gut microbiota of distantly
596 related *Aquatica leii* firefly larvae from China (Zhao et al. 2023). When focusing on key
597 bacterial groups in fireflies, although our single *Pn. consanguineus* group larva did not harbor
598 any mollicute in high abundance, we found that all five late stage *Py. borealis* larvae, sampled in
599 different locations (northern Delaware, eastern New York, southeastern Pennsylvania), harbored
600 moderate to high abundance of the mollicute *Wi. somnilux* (Figure S19 and S20). While the high
601 diversity of larva-associated bacterial communities could easily be explained by environmental
602 acquisition of diverse bacteria from the microbe-rich habitats firefly larvae occur in, such as soil
603 (Torsvik et al. 1990), the presence of the same mollicute species in *Py. borealis* larvae collected
604 from locations separated by hundreds of kilometers further supports our finding that
605 deterministic mechanisms promote the acquisition and persistence of species-specific mollicutes
606 in fireflies. However, it is still unknown whether this mollicute species is acquired from the
607 specific environment of *Py. borealis* larvae. Transmission experiments should be performed in
608 this firefly species to determine the bacterial mode of transmission.

609 Another mystery lies in the fate of symbionts through metamorphosis to adulthood in
610 holometabolous insects. Here, we found that one *Py. borealis* pupa (which was approaching
611 emergence) out of three had detectable, yet low, levels of *Wi. somnilux* (Figure S19 and S20).
612 This bacterium has originally been sampled multiple times in the pupal gut of *Py. angulata* from
613 Maryland (Hackett et al. 1992; Williamson et al. 1990). It thus appears that, at least in

614 *Pyractomena* fireflies, larvae, pupae, and adults associate with species-specific mollicutes, which
615 occasionally appear to persist in the gut during pupation. A drastic reduction in abundance of
616 larva-associated microbes is common during the metamorphosis of holometabolous insects
617 (Hammer and Moran 2019), but the retention of symbionts through pupation has been
618 demonstrated in a few insects before. For example, the fruit fly *Drosophila melanogaster* can
619 harbor high abundance of the mollicute *Spiroplasma* in the gut throughout its lifetime, including
620 during pupation (Anbutsu and Fukatsu 2003). The possible retention of species-specific
621 mollicutes through pupation is yet another indication of the tight, non-random association
622 between these bacteria and their firefly hosts.
623
624 **Insights into the evolutionary history of firefly-associated mollicutes**
625 While the transmission mode of mollicutes in fireflies is still uncertain, we found support that
626 these bacteria non-randomly associate with firefly hosts. Although this is not a prerequisite for
627 being a symbiont specialist (Bourguignon et al. 2018; Cabuslay et al. 2024; Koch et al. 2013),
628 several ancient symbionts are so dependent on their host that they have co-diversified with it
629 (García-Lozano et al. 2024; Hu et al. 2023; Kikuchi et al. 2009; Lo et al. 2003; Zhang et al.
630 2023), forming patterns of co-phylogeny where the symbiont phylogeny tightly matches that of
631 their host. Our phylogenetic analyses (Figure 4; Figure S9), along with previous phylogenomic
632 results (Gupta et al. 2019; Lo et al. 2018), suggest limited evidence for co-cladogenesis between
633 the *Mollicutes* and fireflies, since mollicutes infecting closely related firefly species are rarely
634 each others' closest relatives. For example, the closely related fireflies *Pn. corruscus* and *Pn.*
635 *pyralis* (Catalan et al. 2022) commonly harbored *En. corruscae* and *Wi. lucivorax*, respectively,
636 classified in two distinct bacterial genera (Gupta et al. 2019). These two bacterial species are

637 phylogenetically separated by other bacterial genera with very different lifestyles (e.g., members
638 of the *Mycoplasma* are vertebrate pathogens; Fischer et al. 2012; Waites and Talkington 2004).
639 Even within the mollicute genera that infect fireflies, the preferred host can vary. For example,
640 the *Williamsoniiplasma* genus includes species associated with either lampyrine fireflies or tiger
641 beetles (Tully et al. 1998), which last shared a common ancestor over 300 million years ago
642 (McKenna et al. 2019). Thus, it appears unlikely that mollicute symbionts are the result of
643 ancient domestication in a common host ancestor and conserved through firefly evolution to
644 extant lineages.

645 This incongruence between host and symbiont phylogenies could have two explanations.
646 First, species from the *Mollicutes* may be generalists and associated with multiple host species.
647 These bacteria could switch hosts on multiple occasions over evolutionary time scales, a process
648 that has been documented in other insect-symbiont systems (Sudakaran et al. 2017). While most
649 *Williamsoniiplasma* species appear to specifically associate with one lampyrine species or genus
650 (Figure 5A), the *Wi. luminosum* species was detected at moderate to high abundance in three
651 *Photinus* firefly species (Figure 5A; Figure S11; Fallon et al. 2018; Williamson et al. 1990),
652 suggesting a lower level of host specificity for this mollicute species. This is also supported by
653 *En. ellychniae*, a bacterial strain with a 16S rRNA amplicon sequence almost identical to *En.*
654 *corruscae* (Figure 4), being able to infect *Pn. corruscus* as well as tabanid flies (Wedincamp et
655 al. 1996); and by members of the *Spiroplasma* genus, with single *Spiroplasma* species being
656 detected in different insect species (Hackett et al. 1992; 1996; Stevens et al. 1997; Tully et al.
657 1995). Host switching could conceivably be facilitated by interspecific sexual interactions, a
658 behavior that has been documented among *Photinus* fireflies (Vergara et al. 2025). Future

659 laboratory transplantation experiments could help discern the extent of species-specificity in
660 firefly-mollicute associations.

661 A second possible explanation for the discrepancy in firefly-mollicute phylogenies is that
662 some of these associations may have evolved recently, leading to patterns of co-phylogeny that
663 may only be detectable at lower taxonomic levels, within single mollicute genera, across species
664 or strains. The *Entomoplasma* and *Williamsoniiplasma* genera, for example, may each include
665 additional firefly-associated symbiont species that have to be discovered from yet unscreened
666 firefly species. Given that *Williamsoniiplasma* species can be endemic to single firefly host
667 species (e.g., *Wi. lucivorax* to *Pn. pyralis*), unscreened lampyrine species could plausibly harbor
668 new endemic mollicute taxa. The microbiome of more firefly species should be characterized to
669 determine whether they harbor new mollicute species, sisters to the ones that have already been
670 identified.

671

672 **Conclusion**

673 Through qPCR, deep amplicon sequencing, dissections, phylogenetics, and bioinformatic
674 analyses, we characterized the bacterial communities of fireflies from two common subfamilies
675 in the Northeastern US and evaluated the influence of different factors on bacterial community
676 structure and composition. We found that most firefly species harbor a unique, yet variable
677 microbiome, structured by a combination of stochastic and deterministic forces, and often
678 dominated by members of species-specific mollicutes. Although the intraspecific variation
679 observed in firefly microbiomes likely derives from a combination of stochastic process and
680 geographic, environmental, and host-specific factors, like life stage, age, and sex, the non-
681 random preferential associations with members of the *Mollicutes* raises the possibility that host-

682 specific physiochemical conditions (i.e., habitat filtering mechanisms) contribute to the
683 maintenance and specificity of these bacteria. While the transmission mode of these bacteria is
684 still unknown, given their high abundance when present, mollicutes may play a role in the
685 biology of firefly hosts (Supplementary Text 1). We also occasionally found insect
686 endosymbionts infecting firefly tissues, with yet undetermined roles, but with a hypothetical
687 potential for reproductive manipulation.

688 Altogether, our results suggest that fireflies are associated with species-specific bacteria
689 that are not always present, and that could, hypothetically, be facultative symbionts. In the
690 future, functional characterizations of firefly microbiomes through combinations of targeted
691 experimental antibiotic and fitness assays, genomics, and gene expression analyses will be
692 necessary to better comprehend the nature of the microbe-firefly associations. Firefly biology
693 and ecology can be challenging to study, given the short adult lifespans of most species and the
694 difficulty to rear early developmental stages, especially terrestrial species, in laboratory settings.
695 Improvements of existing methodologies (Buschman 1988; Fallon et al. 2018; Fu and Benno
696 Meyer-Rochow 2013; Ho et al. 2010; McLean et al. 1972; Vaz et al. 2020; Yang et al. 2024)
697 could facilitate the study of their microbiome. This research constitutes a major first step towards
698 a better understanding of the ecology of firefly-associated microbial communities. Future
699 research will shed light on how microbes contribute to the fascinating but puzzling ecology and
700 evolution of fireflies, a group that provides pest control services (Fu and Benno Meyer-Rochow
701 2013) and has high aesthetic value, but is experiencing severe population reductions around the
702 world (Fallon et al. 2018; Lewis et al. 2020).

703

704 **Materials and Methods**

705 **1. Specimen collection and identification.** A total of 344 firefly specimens (*Coleoptera*:
706 *Lampyridae*) were collected opportunistically in the continental United States in 2021, 2022, and
707 2023 (Figure 1A and 1B; Table S1). We captured specimens by hand or with a net, preserved
708 them in > 95% ethanol upon collection, and froze them within the next 72 hours. Firefly
709 specimens were initially identified and sexed via examination of flash patterns, external
710 morphology, and genitalia (Faust 2017; Fender 1970; Green 1956; 1957; McDermott 1967). To
711 verify species identifications, up to 27 specimens from each species and species group were
712 DNA barcoded, for a total of 59 specimens. We extracted DNA as described below and
713 amplified fragments of the universal metazoan cytochrome C oxidase subunit 1 (COI) gene
714 marker using four different primer pairs (Folmer et al. 1994; Geller et al. 2013; Rennstam
715 Rubbmark et al. 2018; Simon et al. 1994; Stanger-Hall et al. 2007). A full description of the
716 barcoding methods is available in Supplementary Text 1 with results shown in Figure S1. DNA
717 barcoding sequences are available as NCBI GenBank files, with accession IDs PV844642-
718 PV844700.

719 The firefly specimens belonged to two subfamilies, four genera, and 12 species or species
720 groups (Figure 1A). We chose to use species group-level classification for three groups
721 (*Photinus consanguineus*, *Pn. marginellus*, and *Photuris versicolor* groups) because (*i*)
722 identification to the species level in the field is challenging in some firefly groups based solely
723 on external morphological characters and flash patterns (e.g., *Photuris*; Faust 2017), (*ii*) while
724 male genitalia morphology can be diagnostic in some firefly groups (e.g., *Photinus*; Green 1956),
725 dissection to extract the aedeagus may compromise the tissues needed for microbiome analyses,
726 and (*iii*) species relationships cannot be resolved with the COI gene for some groups (e.g.
727 *Photuris*, some *Photinus*, *Pyractomena*; Lower et al. 2017; Stanger-Hall and Lloyd 2015), either

728 due to lack of variation in the COI locus, incomplete lineage sorting, or lack of COI sequence in
729 GenBank.
730 Among the collected firefly specimens, 332 were adults, including 104 females, 227
731 males, and one *Lucidota atra* specimen that was not sexed, and 12 were juveniles, with nine
732 larvae and three pupae (Figure 1A). Ten adult beetles from other families were also collected as
733 outgroup specimens. They included three soldier beetles (*Cantharidae*), two tiger beetles
734 (*Cicindelidae*) that were already dead upon collection, three click beetles (*Elateridae*), one net-
735 winged beetle (*Lycidae*), and one fire-colored beetle (*Pyrochroidae*).
736
737 **2. Dissection and DNA extraction.** To study the bacterial communities of fireflies, we extracted
738 DNA from the whole body of 321 specimens. First, fully intact specimens were surface sterilized
739 in 10% bleach, > 95% ethanol, and sterile water. We then flash-froze specimens in empty 1.5 mL
740 tubes by dipping them in liquid nitrogen, before grinding them to powder with a sterile pestle.
741 For each DNA extraction batch (10–23 samples each), a DNA extraction blank sample,
742 consisting of an empty tube, was processed simultaneously with the other samples. DNA was
743 subsequently extracted from flash-frozen, ground samples using the Qiagen DNeasy Blood and
744 Tissue kit (Qiagen, Germantown, MD, USA), following the manufacturer's pretreatment protocol
745 for Gram-positive bacteria.
746 To study the bacterial community of fireflies across tissues, we randomly selected among
747 our preserved specimens 5–6 adult females and males from each of three well-represented firefly
748 taxa (*Photinus corruscus*, *Pn. pyralis*, and the *Pr. versicolor* group; Table S8). We first surface-
749 sterilized the specimens as described above. To isolate tissues, firefly specimens were then
750 dissected in sterile water using bleach-sterilized fine forceps. Separated tissues were placed in

751 pre-chilled 1.5 mL tubes, either filled with 95–100% ethanol or empty, and stored at -80 °C.
752 During the dissections, we separated the entire gut, the reproductive organs, and the lantern (for
753 bioluminescent species) from the rest of the body, which was used as the fourth main tissue (i.e.,
754 "carcass"). To investigate the possibility of endosymbionts (i.e., symbionts residing inside the
755 body or cells of another organism) and/or reproductive manipulators, for a few *Pn. corruscus* and
756 *Pn. pyralis* specimens, we additionally separated some of these main tissues to obtain enlarged
757 fat tissue ("globules"), genitalia, ovaries, hindgut, foregut and midgut, spermatheca,
758 spermatophore-digesting glands, and testes. Dissection blank samples consisted of a firefly leg
759 left in a water droplet on the side of the dissection area. To control for contaminants during
760 dissection, we used a new dissection blank for every one to three dissected specimens. In total,
761 we dissected 33 adult fireflies, resulting in 132 firefly tissue samples and 15 dissection blank
762 samples (Figure S2). We extracted DNA from dissected tissue samples as described above for
763 whole bodies, but without the surface sterilization step.
764 We obtained a total of 497 DNA samples, including 321 whole-body samples, 132
765 dissected tissue samples, 15 dissection blanks, and 29 DNA extraction blank samples.
766
767 **3. Bacterial titer and total DNA quantifications.** Total bacterial titer per sample was obtained
768 through qPCR (Table S10). CT values were generated with a 7300 Real-Time PCR System
769 (Applied Biosystems, Foster City, CA, USA). We used the V4 region of the 16S rRNA gene as
770 the target, amplified using the bacterial universal 515F (5'-GTGCCAGCMGCCGCGGTAA-3')
771 and 806R (5'-GGACTACHVGGGTWTCTAAT-3') primers (Apprill et al. 2015; Caporaso et al.
772 2011; Parada et al. 2016). To obtain absolute bacterial titers measured in 16S rRNA gene copy
773 numbers, we used, in each qPCR plate, a standard curve built on a ten-fold dilution series of a

774 DNA sample with known 16S rRNA gene copy number. Bacterial titers were then corrected
775 using DNA extraction blank and dissection blank samples for tissue samples. Each sample was
776 run in two or three separate technical replicates, consisting of runs on separate qPCR plates. We
777 calculated the average of blank-corrected titers across plate replicates and used these values in
778 downstream statistical analyses. A detailed description of our qPCR procedure can be found in
779 Supplementary Text 1. To normalize bacterial titers and for our Illumina amplicon sequencing
780 decontamination process (see subsection 5.1), we quantified the concentration of extracted DNA
781 per sample using a Qubit 4 fluorometer (Invitrogen, Waltham, MA, USA) and Qubit dsDNA HS
782 and BR kits (Thermo Fisher Scientific, Waltham, MA, USA), with two to three technical
783 replicates per DNA sample.
784
785 **4. Illumina library preparation and sequencing.** A first batch of 20 samples was sequenced at
786 the University of Texas at Arlington Genomic Core Facility in 2021. The library was prepared
787 following Illumina's two-step amplification strategy using primers 341F (5'-
788 CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAATCC-3'), which
789 amplify the V3-V4 hypervariable region of the 16S rRNA gene (Klindworth et al. 2012), as
790 described in (Ravenscraft et al. 2024). Each sample was bidirectionally sequenced on a 600 cycle
791 paired-end run on an Illumina MiSeq platform (Illumina, San Diego, CA, USA). The second
792 batch, containing 477 samples plus one sequencing blank, was sequenced at the Environmental
793 Sample Preparation and Sequencing Facility at Argonne National Laboratory in 2024 (Table
794 S11). The V4 region of the 16S rRNA gene was PCR-amplified with 515F and 806R primers
795 (Caporaso et al. 2012). Amplicons were sequenced on a 300 cycle paired end run in a NextSeq

796 2000 instrument (Illumina) using customized sequencing primers and procedures. For both runs,
797 demultiplexed data were then processed in R (R Core Team 2024) for downstream analysis.
798
799 **5. Bioinformatic pre-treatment.**
800 **5.1. Pre-processing of sequencing data.** For the MiSeq sequencing run, priming sites
801 and poor-quality bases were removed from the 5' and 3' ends of the sequences using the cutadapt
802 v3.2 program (Martin 2011). This step was not necessary for the NextSeq sequences, which did
803 not include priming sites. Then, for each sequencing run, we used the *DADA2* v1.32.0 R package
804 (Callahan et al. 2016) to remove low quality reads, merge paired ends, and infer the bacterial
805 strains present, generating amplicon sequence variants (ASVs). We merged data from the two
806 sequencing runs, performed *de novo* chimera removal, and ran taxonomic classification through
807 the RDP classifier and with a modified SILVA SSU v138.1 training dataset (Quast et al. 2013;
808 Wang et al. 2007). Further details on the pre-processing procedure are available in
809 Supplementary Text 1. Filtered amplicon sequences were exported in fasta format using the
810 *seqinr* v4.2.36 R package (Charif and Lobry 2007) and are available in our data repository
811 (https://doi.org/10.6084/m9.figshare.28306616). Since ASVs from the 20 samples on the MiSeq
812 run were a longer, partially overlapping region of the 16S rRNA and therefore could not be
813 merged with the NextSeq ASVs, we performed all analyses at higher taxonomic levels (e.g.,
814 bacterial genera) and grouped key bacterial taxa phylogenetically (see section 5.2 below).
815 The ASV count table, the taxonomic classification table, and the metadata information
816 (including bacterial titer obtained from qPCR and DNA concentration per sample) were
817 converted into a phyloseq object that contained 99,979 ASVs, using the *phyloseq* v1.48.0 and
818 *speedyseq* v0.5.3.9021 R packages (McLaren 2020; McMurdie and Holmes 2013). Following

819 length filtering (removing 14,757 ASVs that were not between 240 and 430 bp; i.e., 15% of the
820 sequences), removal of ASVs annotated as chloroplasts, mitochondria, or unidentified at the
821 phylum level (removing 6,778 ASVs and 8% of the remaining sequences), and removal of ASVs
822 only obtained from sequencing blank and extraction blank samples (removing 23,724 ASVs and
823 31% of the remaining sequences), we used the *decontam* v1.24.0 R package (Davis et al. 2018),
824 with the stringent "either" frequency or prevalence method to discard contaminant ASVs. With
825 this method, 1,555 ASVs were flagged as contaminants and removed (discarding 2% of the
826 remaining sequences; list available in our data repository:
827 https://doi.org/10.6084/m9.figshare.28306616), resulting in 53,165 ASVs being retained in our
828 dataset. While we removed a high number of ASVs classified as common contaminants with this
829 method (e.g., *Klebsiella*, *Pseudomonas*, *Salmonella*, *Staphylococcus*), we note that it discarded
830 an abundant ASV from the *Acinetobacter* genus; similar studies have also sequenced
831 *Acinetobacter* from fireflies and it is possible this is a common environmental colonist of firefly
832 guts (Green et al. 2021). In addition, a list of bacteria that passed our decontamination pipeline
833 but that could conceivably be contaminants, because they were only relatively abundant in
834 specimens that had a very low bacterial titer, is shown in Table S12 with more details in
835 Supplementary Text 1.

836 Finally, to control for among-sample differences in sequencing depth, our data were
837 rarefied to 30,000 sequences per sample using *phyloseq* (Schloss 2023; 2024; Weiss et al. 2017).
838 Visual inspection of rarefaction curves indicated that observed diversity had plateaued in the
839 samples by this depth. Because rarefying would remove important samples in our dissected
840 tissue dataset, we instead used compositional normalization for tissue comparative analyses,
841 computed with the *microViz* v0.12.4 R package (Barnett et al. 2021). Similarly, rarefied data

842 were not used for our multivariate ordination analyses based on Bray-Curtis distances, since
843 compositional normalization has been shown to be a more robust normalization method for
844 clustering on Bray-Curtis distance matrices (McMurdie and Holmes 2014) and because rarefying
845 caused some species to be removed from our dataset (data from our two *Pyractomena* sp. near
846 *sinuata* whole body specimens did not pass the rarefaction threshold).

847 **5.2. Taxonomic annotation refinement through phylogenetic analyses.** We next
848 performed phylogenetic analyses to refine the taxonomic identification of abundant and
849 prevalent bacterial clades in fireflies (*Mollicutes*; Figure S9), common arthropod endosymbionts
850 (*Rickettsiaceae*, *Rickettsiella*, and *Wolbachia*; Figure S10), and insect pathogens (*Serratia*;
851 Figure S10). For the *Mollicutes*, we used the terminologies from (Gupta et al. 2019), although
852 other authors classify most of the firefly-associated non-*Spiroplasma* mollicutes as the
853 *Entomoplasma* genus (Balish et al. 2019; Gasparich and Kuo 2019; Lo et al. 2018; Yan et al.
854 2024; but see Arahal et al. 2022; Gupta et al. 2018; Gupta and Oren 2020). Because our data
855 came from two sequencing batches that used two different amplicon lengths, hence classifying
856 identical variants as two different ASVs, we used phylogenetic analyses to group together ASVs
857 that were likely from the same microorganism but were classified as different variants due to
858 different amplicon sizes. We use the term "bacterial phylotype" hereafter in this section,
859 approximately equivalent to bacterial species, to refer to this classification, which we performed
860 only for the five aforementioned bacterial clades (*Mollicutes*, *Rickettsiaceae*, *Rickettsiella*,
861 *Serratia*, and *Wolbachia*).

862 To build these phylogenies, we first selected the 16S rRNA gene amplicon sequence of
863 the most abundant ASVs (those numbered from sv0001 to sv1500, sorted by abundance in our
864 dataset) that were classified as either from the *Mollicutes* class (i.e., *Entomoplasmatales* and

865 *Mycoplasmatales* orders *sensu* SILVA database), from the non-*Anaplasmataceae Rickettsiales*
866 order (i.e., including the *Rickettsiaceae* family, unidentified *Rickettsiales*, and *Candidatus*
867 Hepatincola), or from one of the focal bacterial genera (*Rickettsiella*, *Serratia*, and *Wolbachia*).
868 We used these sequences in NCBI BLASTn runs and downloaded the 16S rRNA gene sequence
869 of the closest matches. We also downloaded the sequences of reference, representative bacterial
870 species from NCBI. For the *Mollicutes*, representative amplicon sequences of each of the firefly-
871 associated bacterial species found in Green et al. (2021) were also used, as well as a few insect-
872 associated mollicute sequences from Funaro et al. (2011). With all these sequences, we produced
873 Clustal Omega (Sievers et al. 2011) multiple sequence alignments in SeaView v5.0.5 (Gouy et
874 al. 2009), which were used to build maximum likelihood phylogenies using RAxML Blackbox
875 (Stamatakis 2014), implemented in the CIPRES Science Gateway (Miller et al. 2010), with 999
876 bootstrap iterations. All phylogenetic trees were visualized and manually annotated in iTOL
877 (Letunic and Bork 2016). Further details on these phylogenetic methods can be found in
878 Supplementary Text 1.
879 Finally, we manually edited the *phyloseq* taxonomy table to account for this taxonomic
880 refinement (available as a Data Repository: https://doi.org/10.6084/m9.figshare.28306616).
881 Except for analyses on alpha diversity metrics and specific mentions of analyses ran at the
882 bacterial order or genus levels, all downstream analyses were run on microbiome data aggregated
883 at the genus level, except for the aforementioned groups we refined (*Mollicutes*, *Rickettsiaceae*,
884 *Rickettsiella*, *Serratia*, and *Wolbachia*) which were aggregated at the "phylotype" level (Figure
885 S2). This combination of genus and phylotype level classification is hereafter referred to as
886 "taxon" level. Data was aggregated at the bacterial order, genus, and taxon levels using the
887 *microViz* R package.

888

889 **6. Bioinformatic analyses.**

890 **6.1. Stochasticity analyses**. To infer the relative importance of stochastic *vs.*
891 deterministic processes contributing to microbiome assembly, we calculated the normalized
892 stochasticity ratio (i.e., NST index) for each firefly species that had more than two specimens in
893 our dataset through implementation of null-models using the *NST* R package v3.1.9 (Ning et al.
894 2019). An index value between 0.5 and 1 suggests that stochastic processes shape community
895 assembly, whereas a value between 0 and 0.5 indicates higher influence of deterministic
896 processes. Next, to infer the contribution of specific bacterial groups, we tested how the removal
897 of either all the mollicutes, all putative endosymbionts (*Ca.* Hepatincola, *Rickettsia*, *Rickettsiella*,
898 and *Wolbachia*), or both, from the microbiome data of each firefly species affects the NST index
899 value. We generated 95% confidence intervals via bootstrapping and tested NST variation
900 between the unaltered microbiome of firefly species *vs.* that following removal of the
901 aforementioned bacterial groups through pairwise, two-tailed Wilcoxon signed-rank statistical
902 tests (Table S2).

903 **6.2. Alpha and beta diversity**. To assess patterns of diversity, richness, evenness,
904 dominance, and rarity in bacterial communities from fireflies, indices of alpha diversity were
905 calculated on rarefied data with non-aggregated (ASV level) taxonomic tables, using the
906 *microbiome* v1.26.0 R package (Lahti and Shetty 2017) (Table S4). To compare bacterial
907 composition across firefly species, genera, subfamilies, and diverse factors, we used beta
908 diversity metrics, first running ordinations on Bray-Curtis distance matrices computed from non-
909 rarefied, proportional abundance data using nonmetric multidimensional scaling (NMDS)
910 analyses computed with *phyloseq*, to infer similarities among fireflies from different taxa, diets,

911 adult use of bioluminescence, regions, and tissues. To test how different factors were correlated
912 with bacterial composition across fireflies, we then ran permutational multivariate analysis of
913 variance (PERMANOVAs) on rarefied, taxon-aggregated data, with the *vegan* v2.6.4 R package
914 (Oksanen et al. 2016), and pairwise PERMANOVAs as post hoc tests using the *pairwiseAdonis*
915 v0.4.1 R package (Martinez 2020).

916 **6.3. Host relatedness, geographic distance, and microbiome dissimilarity**. The impact
917 of firefly host phylogeny on bacterial community dissimilarity was assessed using Mantel tests.
918 We first generated a host phylogeny using five previously sequenced firefly genes (Catalan et al.
919 2022; Martin et al. 2017; Sander and Hall 2015; Stanger-Hall and Lloyd 2015; Stanger-Hall et al.
920 2007) (Figure S1; Supplementary Text 1). We then used the *ape* v5.8 R package (Paradis et al.
921 2004) to generate a phylogenetic distance matrix from our tree with the cophenetic.phylo()
922 function. We next produced a matrix of geographic distance among collected firefly specimens
923 calculated from latitude and longitude decimal variables at collection using the *sf* v1.0.17 R
924 package (Pebesma 2018). As proxies for microbiome dissimilarity, we generated two pairwise
925 distance matrices using the Bray-Curtis distance from rarefied relative bacterial abundance
926 between firefly specimens and the Bray-Curtis binary distance from bacterial presence/absence
927 (equivalent of Sørensen-Dice distance) between firefly specimens. Bacterial presence was
928 defined as any bacterial taxon with at least 1% relative abundance in a specimen and absence as
929 less than 1% relative abundance in that specimen. Because the number of pairwise comparisons
930 was not computationally tractable, we applied a filter to our dataset, keeping only bacterial taxa
931 that had more than 0.5% relative abundance in at least 5% of all firefly specimens. These
932 microbiome dissimilarity matrices were compared to a matrix of phylogenetic distance and to

933 geographic distances between firefly specimens through partial Mantel tests using the *vegan* R
934 package, allowing us to control for host phylogeny and geography, respectively.

935 **6.4. Contribution of firefly ecological traits to bacterial abundance and diversity**. We
936 tested the influence of adult bioluminescence, diet, and body size of firefly species on bacterial
937 abundance and diversity, as well as correlations between bacterial abundance and diversity
938 metrics via generalized least square analyses with the *nlme* v3.1.164 R package (Pinheiro et al.
939 2024). To account for the effects of phylogenetic nonindependence in comparisons of firefly
940 species-level covariates, we used phylogenetic generalized least square (PGLS) analyses, fitting
941 a Brownian motion model of evolution. The firefly phylogeny was obtained as described in
942 section 6.3 and Supplementary Text 1 (Figure S1). Bacterial titer and diversity indices were
943 averaged per firefly species. We also averaged body size (i.e., total adult male body length) per
944 firefly species or species groups using the ranges from Faust (2017), Fender (1970), Green
945 (1956, 1957), and Lloyd (1969).

946 **6.5. Bacterial composition and differential abundance analysis**. To test whether single
947 bacteria vary in their abundance across fireflies and factors, we used the *MaAsLin2* v1.15.1 R
948 package (Mallick et al. 2021), fitting our rarefied (or non-rarefied for tissue analyses) data to
949 negative binomial distributions and controlling for random effects (see section 6.7. Statistical
950 tests) in multiple generalized linear mixed models. To account for variables with unbalanced
951 discrete levels (e.g., species with high *vs.* low number of specimens sampled), when more than
952 two discrete levels were present in our fixed effect(s), we ran the same *MaAsLin2* models
953 multiple times, changing the reference level each time (Table S5, S6, S7, and S9). We filtered
954 out bacterial taxa detected in less than 1% of all firefly samples being tested, unless the test was
955 run on groups with very low sample size (prevalence threshold lowered to 0.1% instead).

956 **6.6. Bacterial co-occurrence analysis**. To investigate positive and negative co-
957 occurrence patterns among bacteria in adult fireflies, we ran co-occurrence analyses, within
958 firefly species that had more than eight adult individuals in our dataset (i.e., excluding *Lu. atra*,
959 *Pn. consanguineus* group, *Pn. cookii*, and *Py. borealis*) and grouping all *Photuris* together. We
960 used both the *cooccur* v1.3 and *CooccurrenceAffinity* v1.0 R packages (Griffith et al. 2016;
961 Mainali and Slud 2022). A filter was applied before analyses to obtain a manageable number of
962 bacterial taxa to be tested per firefly species. Bacterial taxa were considered present when they
963 had at least 1% relative abundance (absent otherwise) in a specimen and were kept in these
964 analyses when they had more than 10,000 reads—or 20,000 reads for the *Photuris*—in one or
965 more samples for a given firefly species or species group.
966 **6.7. Statistical tests**. In addition to the tests described in the previous sections,
967 differences in alpha diversity and qPCR titers across hosts and diverse environmental factors
968 were assessed using linear mixed models (LMM) with the *lme4* v1.1.35.5 R package (Bates et al.
969 2015), which allowed us to control for random effects that may otherwise bias our interpretation
970 of statistical tests. Some key factors were always kept as random effects in our models because
971 they had a strong influence on the microbiome of fireflies in our datasets. These random factors
972 included the year of collection, the geographic location of sampling (either address, longitude,
973 latitude, or region), and collection day or month of year (a rough proxy for firefly age in many
974 species). When they were not among the fixed effects in our models, host sex (which included
975 developmental stages) and species were used as random effects or covariates. The importance of
976 other random effects in our models was previously tested through bidirectional model selection
977 with likelihood ratio tests and comparing model's AICs.

We tested the distribution of the residuals, percentage of the dispersion of the data, the presence of outliers, and zero-inflation using the *performance* v0.12.3 and *DHARMa* v0.4.6 R packages (Hartig 2018; Lüdecke et al. 2021). Our models were often flagged as poor fits due to zero-inflation, despite normalization and log10 transformation. When this was the case, we fitted our data to a negative binomial distribution in generalized LMMs (GLMMs) using *lme4*. We next performed pairwise post hoc tests using the *emmeans* v1.10.4 R package (Lenth 2022) to compute the estimated marginal means (Searle et al. 1980). In doing so, we applied an asymptotic degrees-of-freedom method, thus invoking *z*-statistics, which are computationally faster and recommended with larger sample sizes. A description of all LMMs, GLMMs, PERMANOVAs, pairwise PERMANOVAs, Mantel tests, OLS, and PGLS models is available in Table S3.

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

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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.

Dada 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 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.

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Conflict of Interest Statement

1023 The authors declare that they have no conflict of interest.

1024

1025 ## References

1026 Alizon, Samuel, Jacobus C. de Roode, and Yannis Michalakis. 2013. "Multiple Infections and
1027 the Evolution of Virulence." *Ecology Letters* 16 (4): 556–67.
1028 https://doi.org/10.1111/ele.12076.

1029 Anbutsu, Hisashi, and Takema Fukatsu. 2003. "Population Dynamics of Male-Killing and Non-
1030 Male-Killing Spiroplasmas in *Drosophila Melanogaster*." *Applied and Environmental*
1031 *Microbiology* 69 (3): 1428–34. https://doi.org/10.1128/AEM.69.3.1428-1434.2003.

1032 Apprill, Amy, Sean McNally, Rachel Parsons, and Laura Weber. 2015. "Minor Revision to V4
1033 Region SSU rRNA 806R Gene Primer Greatly Increases Detection of SAR11
1034 Bacterioplankton." *Aquatic Microbial Ecology* 75 (2): 129–37.

1035 Arahal, David R., Hans-Jürgen Busse, Carolee T. Bull, et al. 2022. "Judicial Opinions 112–122."
1036 In *International Journal of Systematic and Evolutionary Microbiology*, vol. 72. no. 8.
1037 Microbiology Society. https://doi.org/10.1099/ijsem.0.005481.

1038 Balish, Mitchell, Assunta Bertaccini, Alain Blanchard, et al. 2019. "Recommended Rejection of
1039 the Names Malacoplasma Gen. Nov., Mesomycoplasma Gen. Nov., Metamycoplasma
1040 Gen. Nov., Metamycoplasmataceae Fam. Nov., Mycoplasmoidaceae Fam. Nov.,
1041 Mycoplasmoidales Ord. Nov., Mycoplasmoides Gen. Nov., Mycoplasmopsis Gen. Nov.
1042 [Gupta, Sawnani, Adeolu, Alnajar and Oren 2018] and All Proposed Species Comb. Nov.
1043 Placed Therein." In *International Journal of Systematic and Evolutionary Microbiology*,
1044 vol. 69. no. 11. Microbiology Society. https://doi.org/10.1099/ijsem.0.003632.

1045 Barnett, David J. m, Ilja C. w Arts, and John Penders. 2021. "microViz: An R Package for
1046 Microbiome Data Visualization and Statistics." *Journal of Open Source Software* 6 (63):
1047 3201. https://doi.org/10.21105/joss.03201.
1048 Bates, Douglas, Martin Mächler, Ben Bolker, and Steve Walker. 2015. "Fitting Linear Mixed-
1049 Effects Models Using Lme4." Articles. *Journal of Statistical Software* 67 (1): 1.
1050 https://doi.org/10.18637/jss.v067.i01.
1051 Baumann, Paul. 2005. "BIOLOGY OF BACTERIOCYTE-ASSOCIATED ENDOSYMBIONTS
1052 OF PLANT SAP-SUCKING INSECTS." In *Annual Review of Microbiology*, vol. 59. no.
1053 Volume 59, 2005. Annual Reviews.
1054 https://doi.org/10.1146/annurev.micro.59.030804.121041.
1055 Bäumler, Andreas, and Ferric C. Fang. 2013. "Host Specificity of Bacterial Pathogens." *Cold*
1056 *Spring Harbor Perspectives in Medicine* 3 (12).
1057 https://doi.org/10.1101/cshperspect.a010041.
1058 Bourguignon, Thomas, Nathan Lo, Carsten Dietrich, et al. 2018. "Rampant Host Switching
1059 Shaped the Termite Gut Microbiome." *Current Biology* 28 (4): 4.
1060 https://doi.org/10.1016/j.cub.2018.01.035.
1061 Buschman, Lawrent L. 1984a. "Biology of the Firefly Pyractomena Lucifera (Coleoptera:
1062 Lampyridae)." *The Florida Entomologist* 67 (4): 4. JSTOR.
1063 https://doi.org/10.2307/3494462.
1064 Buschman, Lawrent L. 1984b. "Larval Biology and Ecology of Photuris Fireflies (Lampyridae:
1065 Coleoptera) in Northcentral Florida." *Journal of the Kansas Entomological Society* 57
1066 (1): 1. JSTOR.

1067 Buschman, Lawrent L. 1988. "Larval Development and Its Photoperiodic Control in the Firefly
1068 Pyractomena Lucifera (Coleoptera: Lampyridae)." *Annals of the Entomological Society*
1069 *of America* 81 (1): 1. https://doi.org/10.1093/aesa/81.1.82.
1070 Cabuslay, Christian, John T. Wertz, Benoît Béchade, et al. 2024. "Domestication and
1071 Evolutionary Histories of Specialized Gut Symbionts across Cephalotine Ants."
1072 *Molecular Ecology* 33 (15): e17454. https://doi.org/10.1111/mec.17454.
1073 Callahan, Benjamin J, Paul J McMurdie, Michael J Rosen, Andrew W Han, Amy Jo A Johnson,
1074 and Susan P Holmes. 2016. "DADA2: High-Resolution Sample Inference from Illumina
1075 Amplicon Data." *Nature Methods* 13 (7): 581–83. https://doi.org/10.1038/nmeth.3869.
1076 Caporaso, J. Gregory, Christian L. Lauber, William A. Walters, et al. 2011. "Global Patterns of
1077 16S rRNA Diversity at a Depth of Millions of Sequences per Sample." *Proceedings of*
1078 *the National Academy of Sciences* 108 (supplement_1): supplement_1.
1079 https://doi.org/10.1073/pnas.1000080107.
1080 Caporaso, J Gregory, Christian L Lauber, William A Walters, et al. 2012. "Ultra-High-
1081 Throughput Microbial Community Analysis on the Illumina HiSeq and MiSeq
1082 Platforms." *The ISME Journal* 6 (8): 1621–24. https://doi.org/10.1038/ismej.2012.8.
1083 Caragata, Eric P., Edwige Rancès, Lauren M. Hedges, et al. 2013. "Dietary Cholesterol
1084 Modulates Pathogen Blocking by Wolbachia." *PLoS Pathogens* 9 (6): 1–9.
1085 https://doi.org/10.1371/journal.ppat.1003459.
1086 Carpenter, Melissa, Linyao Peng, Andrew H. Smith, et al. 2021. "Frequent Drivers, Occasional
1087 Passengers: Signals of Symbiont-Driven Seasonal Adaptation and Hitchhiking in the Pea
1088 Aphid, Acyrthosiphon Pisum." *Insects* 12 (9). https://doi.org/10.3390/insects12090805.

1089 Cassells, Maria D., Sophie Labaude, and Christine T. Griffin. 2024. "Beware Glowing Cadavers:
1090 Bioluminescence of Nematode Symbiont Photorhabdus Protects Nematode-Infected Host
1091 Cadavers from Nocturnal Scavengers." *Frontiers in Ecology and Evolution* 11.
1092 https://doi.org/10.3389/fevo.2023.1264251.
1093 Catalan, Ana, Sebastian Höhna, Sarah E. Lower, and Pablo Duchen. 2022. "Inferring the
1094 Demographic History of the North American Firefly Photinus Pyralis." *Journal of*
1095 *Evolutionary Biology* 35 (11): 1488–99. https://doi.org/10.1111/jeb.14094.
1096 Catalán, Ana, Claudia María Pérez Archila, Adriana María Echeverría Méndez, et al. 2025.
1097 "Filling the Gap in the Neotropics: New Firefly Species Provide Insights into Central
1098 American Biodiversity." *bioRxiv*, January 1, 2025.02.20.639311.
1099 https://doi.org/10.1101/2025.02.20.639311.
1100 Chandler, James Angus, Jenna Morgan Lang, Srijak Bhatnagar, Jonathan A. Eisen, and Artyom
1101 Kopp. 2011. "Bacterial Communities of Diverse Drosophila Species: Ecological Context
1102 of a Host–Microbe Model System." *PLoS Genetics* 7 (9): 1–18.
1103 https://doi.org/10.1371/journal.pgen.1002272.
1104 Charif, Delphine, and Jean R. Lobry. 2007. "SeqinR 1.0-2: A Contributed Package to the R
1105 Project for Statistical Computing Devoted to Biological Sequences Retrieval and
1106 Analysis." In *Structural Approaches to Sequence Evolution: Molecules, Networks,*
1107 *Populations*, edited by Ugo Bastolla, Markus Porto, H. Eduardo Roman, and Michele
1108 Vendruscolo. Springer Berlin Heidelberg. https://doi.org/10.1007/978-3-540-35306-
1109 5_10.

1110 Coyte, Katharine Z., Chitong Rao, Seth Rakoff-Nahoum, and Kevin R. Foster. 2021. "Ecological
1111 Rules for the Assembly of Microbiome Communities." *PLOS Biology* 19 (2): 1–19.
1112 https://doi.org/10.1371/journal.pbio.3001116.
1113 Davis, Nicole M., Diana M. Proctor, Susan P. Holmes, David A. Relman, and Benjamin J.
1114 Callahan. 2018. "Simple Statistical Identification and Removal of Contaminant
1115 Sequences in Marker-Gene and Metagenomics Data." *Microbiome* 6 (1): 226.
1116 https://doi.org/10.1186/s40168-018-0605-2.
1117 Degregori, Samuel, Nina M. D. Schiettekatte, Jordan M. Casey, et al. 2024. "Host Diet Drives
1118 Gut Microbiome Convergence between Coral Reef Fishes and Mammals." *Molecular*
1119 *Ecology* 33 (19): e17520. https://doi.org/10.1111/mec.17520.
1120 Deyrup, Stephen T., Riley G. Risteen, Kathareeya K. Tonyai, et al. 2017. "Escape into Winter:
1121 Does a Phenological Shift by Ellychnia Corrusca (Winter Firefly) Shield It from a
1122 Specialist Predator (Photuris)?" *Northeastern Naturalist* 24 (sp7).
1123 https://doi.org/10.1656/045.024.s717.
1124 Dillon, R.J., and V.M. Dillon. 2003. "THE GUT BACTERIA OF INSECTS: Nonpathogenic
1125 Interactions." *Annual Review of Entomology* 49 (1): 1.
1126 https://doi.org/10.1146/annurev.ento.49.061802.123416.
1127 Fallon, Candace E., Anna C. Walker, Sara Lewis, et al. 2021. "Evaluating Firefly Extinction
1128 Risk: Initial Red List Assessments for North America." *PLoS ONE* 16 (11): 11.
1129 https://doi.org/10.1371/journal.pone.0259379.
1130 Fallon, Timothy R, Sarah E Lower, Ching-Ho Chang, et al. 2018. "Firefly Genomes Illuminate
1131 Parallel Origins of Bioluminescence in Beetles." *eLife* 7 (October): e36495.
1132 https://doi.org/10.7554/eLife.36495.

Faust, L.F. 2017. *Fireflies, Glow-Worms, and Lightning Bugs: Identification and Natural History of the Fireflies of the Eastern and Central United States and Canada*. Wormsloe Foundation Nature Book. University of Georgia Press. https://books.google.com/books?id=1HELDgAAQBAJ.

Faust, Lynn. 2012. "Fireflies in the Snow: Observations on Two Early-Season Arboreal Fireflies Ellychnia Corrusca and Pyractomena Borealis." *Lampyrid* 2: 48–71.

Faust, Lynn, and Hugh Faust. 2014. "The Occurrence and Behaviors of North American Fireflies (Coleoptera: Lampyridae) on Milkweed, Asclepias Syriaca L." *The Coleopterists Bulletin* 68 (2): 2. https://doi.org/10.1649/0010-065X-68.2.283.

Fender, Kenneth M. 1970. "Ellychnia of Western North America (Coleoptera: Lampyridae)." *Northwest Science* 44 (1): 31–43.

FireflyAtlas.org. 2025. "Firefly Species Checklist of the USA and Canada - Xerces Society." https://www.fireflyatlas.org/firefly-species/firefly-species-checklist.

Fischer, Anne, Beth Shapiro, Cecilia Muriuki, et al. 2012. "The Origin of the 'Mycoplasma Mycoides Cluster' Coincides with Domestication of Ruminants." *PLOS ONE* 7 (4): 1–6. https://doi.org/10.1371/journal.pone.0036150.

Folmer, O., M. Black, W. Hoeh, R. Lutz, and R. Vrijenhoek. 1994. "DNA Primers for Amplification of Mitochondrial Cytochrome c Oxidase Subunit I from Diverse Metazoan Invertebrates." *Molecular Marine Biology and Biotechnology* 3 (5): 5. 7881515.

Fu, Xinhua, and V. Benno Meyer-Rochow. 2013. "Larvae of the Firefly Pyrocoelia Pectoralis (Coleoptera: Lampyridae) as Possible Biological Agents to Control the Land Snail Bradybaena Ravida." *Biological Control* 65 (2): 2. https://doi.org/10.1016/j.biocontrol.2013.02.005.

1156 Funaro, Colin F., Daniel J. C. Kronauer, Corrie S. Moreau, Benjamin Goldman-Huertas, Naomi
1157 E. Pierce, and Jacob A. Russell. 2011. "Army Ants Harbor a Host-Specific Clade of
1158 Entomoplasmatales</Em> Bacteria." *Applied and Environmental Microbiology* 77
1159 (1): 1. https://doi.org/10.1128/AEM.01896-10.
1160 Furman, Ori, Liat Shenhav, Goor Sasson, et al. 2020. "Stochasticity Constrained by
1161 Deterministic Effects of Diet and Age Drive Rumen Microbiome Assembly Dynamics."
1162 *Nature Communications* 11 (1): 1904. https://doi.org/10.1038/s41467-020-15652-8.
1163 García-Lozano, Marleny, Christine Henzler, Miguel Ángel González Porras, et al. 2024.
1164 "Paleocene Origin of a Streamlined Digestive Symbiosis in Leaf Beetles." *Current*
1165 *Biology* 34 (8): 1621-1634.e9. https://doi.org/10.1016/j.cub.2024.01.070.
1166 Gasparich, Gail E., and Chih-Horng Kuo. 2019. "Genome Analysis-Based Union of the Genus
1167 Mesoplasma with the Genus Entomoplasma." In *International Journal of Systematic and*
1168 *Evolutionary Microbiology*, No. 9, vol. 69. no. 9. Microbiology Society.
1169 https://doi.org/10.1099/ijsem.0.003548.
1170 Geller, J., C. Meyer, M. Parker, and H. Hawk. 2013. "Redesign of PCR Primers for
1171 Mitochondrial Cytochrome c Oxidase Subunit I for Marine Invertebrates and Application
1172 in All-Taxa Biotic Surveys." *Molecular Ecology Resources* 13 (5): 851–61.
1173 https://doi.org/10.1111/1755-0998.12138.
1174 Gofton, Alexander W., Charlotte L. Oskam, Nathan Lo, et al. 2015. "Inhibition of the
1175 Endosymbiont 'Candidatus Midichloria Mitochondrii' during 16S rRNA Gene Profiling
1176 Reveals Potential Pathogens in Ixodes Ticks from Australia." *Parasites & Vectors* 8 (1):
1177 345. https://doi.org/10.1186/s13071-015-0958-3.

1178 Gouy, Manolo, Stéphane Guindon, and Olivier Gascuel. 2009. "SeaView Version 4: A
1179 Multiplatform Graphical User Interface for Sequence Alignment and Phylogenetic Tree
1180 Building." *Molecular Biology and Evolution* 27 (2): 2.
1181 https://doi.org/10.1093/molbev/msp259.
1182 Green, Emily A., Scott R. Smedley, and Jonathan L. Klassen. 2021. "North American Fireflies
1183 Host Low Bacterial Diversity." *Microbial Ecology* 82 (3): 3.
1184 https://doi.org/10.1007/s00248-021-01718-7.
1185 Green, John Wagener. 1956. "Revision of the Nearctic Species of Photinus:(Lampyridae:
1186 Coleoptera)." *Proceedings of the California Academy of Sciences* 28: 561–613.
1187 Green, JOHN WAGONER. 1957. "Revision of the Nearctic Species of Pyractomena
1188 (Coleoptera: Lampyridae)." *The Wasmann Journal of Biology* 15 (2): 237–84.
1189 Griffith, Daniel M., Joseph A. Veech, and Charles J. Marsh. 2016. "Cooccur: Probabilistic
1190 Species Co-Occurrence Analysis in R." Code Snippets. *Journal of Statistical Software* 69
1191 (2): 1–17. https://doi.org/10.18637/jss.v069.c02.
1192 Gu, Xinyue, Perran A. Ross, Alex Gill, et al. 2023. "A Rapidly Spreading Deleterious Aphid
1193 Endosymbiont That Uses Horizontal as Well as Vertical Transmission." *Proceedings of*
1194 *the National Academy of Sciences* 120 (18): e2217278120.
1195 https://doi.org/10.1073/pnas.2217278120.
1196 Gupta, Radhey S., and Aharon Oren. 2020. "Necessity and Rationale for the Proposed Name
1197 Changes in the Classification of Mollicutes Species. Reply to: 'Recommended Rejection
1198 of the Names Malacoplasma Gen. Nov., Mesomycoplasma Gen. Nov., Metamycoplasma
1199 Gen. Nov., Metamycoplasmataceae Fam. Nov., Mycoplasmoidaceae Fam. Nov.,
1200 Mycoplasmoidales Ord. Nov., Mycoplasmoides Gen. Nov., Mycoplasmopsis Gen. Nov.

[Gupta, Sawnani, Adeolu, Alnajar and Oren 2018] and All Proposed Species Comb. Nov. Placed Therein', by M. Balish et al. (Int J Syst Evol Microbiol, 2019;69:3650–3653)." In *International Journal of Systematic and Evolutionary Microbiology*, vol. 70. no. 2. Microbiology Society. https://doi.org/10.1099/ijsem.0.003869.

Gupta, Radhey S., Sahil Sawnani, Mobolaji Adeolu, Seema Alnajar, and Aharon Oren. 2018. "Phylogenetic Framework for the Phylum Tenericutes Based on Genome Sequence Data: Proposal for the Creation of a New Order Mycoplasmoidales Ord. Nov., Containing Two New Families Mycoplasmoidaceae Fam. Nov. and Metamycoplasmataceae Fam. Nov. Harbouring Eperythrozoon, Ureaplasma and Five Novel Genera." *Antonie van Leeuwenhoek* 111 (9): 1583–630. https://doi.org/10.1007/s10482-018-1047-3.

Gupta, Radhey S., Jeen Son, and Aharon Oren. 2019. "A Phylogenomic and Molecular Markers Based Taxonomic Framework for Members of the Order Entomoplasmatales: Proposal for an Emended Order Mycoplasmatales Containing the Family Spiroplasmataceae and Emended Family Mycoplasmataceae Comprised of Six Genera." *Antonie van Leeuwenhoek* 112 (4): 4. https://doi.org/10.1007/s10482-018-1188-4.

Hackett, K. J., R. F. Whitcomb, F. E. French, et al. 1996. "Spiroplasma Corruscae Sp. Nov., from a Firefly Beetle (Coleoptera: Lampyridae) and Tabanid Flies (Diptera: Tabanidae)." In *International Journal of Systematic and Evolutionary Microbiology*, vol. 46. no. 4. Microbiology Society. https://doi.org/10.1099/00207713-46-4-947.

Hackett, K. J., R. F. Whitcomb, J. G. Tully, et al. 1992. "Lampyridae (Coleoptera): A Plethora of Mollicute Associations." *Microbial Ecology* 23 (2): 2. https://doi.org/10.1007/BF00172639.

1223 Halliday, Fletcher W., Elle M. Barnes, Miriam N. Ojima, and Isabelle Stiver. 2024. "On the Hunt
1224 for Facilitation in Symbiont Communities." *Trends in Ecology & Evolution* 39 (9): 793–
1225 96. https://doi.org/10.1016/j.tree.2024.07.009.
1226 Hammer, Tobin J. 2024. "Why Do Hosts Malfunction without Microbes? Missing Benefits
1227 versus Evolutionary Addiction." *Trends in Microbiology* 32 (2): 132–41.
1228 https://doi.org/10.1016/j.tim.2023.07.012.
1229 Hammer, Tobin J., and Nancy A. Moran. 2019. "Links between Metamorphosis and Symbiosis
1230 in Holometabolous Insects." *Philosophical Transactions of the Royal Society B:*
1231 *Biological Sciences* 374 (1783): 1783. https://doi.org/10.1098/rstb.2019.0068.
1232 Hartig, F. 2018. *DHARMa: Residual Diagnostics for Hierarchical (Multi-Level/Mixed)*
1233 *Regression Models. R Package Version 0.4.6.* V. 0.4.6. Released. https://CRAN.R-
1234 project.org/package=DHARMa.
1235 Hendrycks, Wouter, Nele Mullens, Jackline Bakengesa, et al. 2025. "Deterministic and
1236 Stochastic Effects Drive the Gut Microbial Diversity in Cucurbit-Feeding Fruit Flies
1237 (Diptera, Tephritidae)." *PLoS One* 20 (1): 1–20.
1238 https://doi.org/10.1371/journal.pone.0313447.
1239 Hess, WALTER N. 1920. "NOTES ON THE BIOLOGY OF SOME COMMON
1240 LAMPYRIDAE." *The Biological Bulletin* 38 (2): 2. https://doi.org/10.2307/1536232.
1241 Ho, Jen-Zon, Pi-Hei Chiang, Chia-Hsiung Wu, and Ping-Shih Yang. 2010. "Life Cycle of the
1242 Aquatic Firefly Luciola Ficta (Coleoptera: Lampyridae)." *Journal of Asia-Pacific*
1243 *Entomology* 13 (3): 3. https://doi.org/10.1016/j.aspen.2010.03.007.
1244 Höhna, Sebastian, Sarah E Lower, Pablo Duchen, and Ana Catalán. 2024. "Robustness of
1245 Divergence Time Estimation despite Gene Tree Estimation Error: A Case Study of

Fireflies (Coleoptera: Lampyridae).” *Systematic Biology*, November, syae065. https://doi.org/10.1093/sysbio/syae065.

Hosen, Jacob D., Catherine M. Febria, Byron C. Crump, and Margaret A. Palmer. 2017. “Watershed Urbanization Linked to Differences in Stream Bacterial Community Composition.” *Frontiers in Microbiology* 8. https://doi.org/10.3389/fmicb.2017.01452.

Hu, Yi, Catherine L. D’Amelio, Benoît Béchade, et al. 2023. “Partner Fidelity and Environmental Filtering Preserve Stage-Specific Turtle Ant Gut Symbioses for over 40 Million Years.” *Ecological Monographs* 93 (1): 1. https://doi.org/10.1002/ecm.1560.

iNaturalist community. 2025. *Observations of Ellychnia Corrusca. Exported from Https://Www.Inaturalist.Org on 25 January 2025*.

Itoh, Hideomi, Seonghan Jang, Kazutaka Takeshita, et al. 2019. “Host–Symbiont Specificity Determined by Microbe–Microbe Competition in an Insect Gut.” *Proceedings of the National Academy of Sciences* 116 (45): 45. https://doi.org/10.1073/pnas.1912397116.

Jeong, Gilsang, Taehwa Kang, Haechul Park, et al. 2009. “Wolbachia Infection in the Korean Endemic Firefly, Luciola Unmunsana (Coleoptera: Lampyridae).” *Journal of Asia-Pacific Entomology* 12 (1): 1. https://doi.org/10.1016/j.aspen.2008.11.001.

Jeyaprakash, A., and M. A. Hoy. 2000. “Long PCR Improves Wolbachia DNA Amplification: Wsp Sequences Found in 76% of Sixty-Three Arthropod Species.” *Insect Molecular Biology* 9 (4): 4. https://doi.org/10.1046/j.1365-2583.2000.00203.x.

Jones, B. W., and M. K. Nishiguchi. 2004. “Counterillumination in the Hawaiian Bobtail Squid, Euprymna Scolopes Berry (Mollusca: Cephalopoda).” *Marine Biology* 144 (6): 6. https://doi.org/10.1007/s00227-003-1285-3.

1268 Jones, Eric W., Jean M. Carlson, David A. Sivak, and William B. Ludington. 2022. "Stochastic
1269 Microbiome Assembly Depends on Context." *Proceedings of the National Academy of*
1270 *Sciences* 119 (7): e2115877119. https://doi.org/10.1073/pnas.2115877119.
1271 Karasov, William H., and Angela E. Douglas. 2013. "Comparative Digestive Physiology." In
1272 *Compr Physiol*, Terjung R., vol. 3. https://doi.org/10.1002/cphy.c110054.
1273 Keller, O. 2025. "The Lampyridae of the World Database." https://lampyridae.world/.
1274 Kikuchi, Y., T. Hosokawa, N. Nikoh, X. -. Y. Meng, Y. Kamagata, and T. Fukatsu. 2009. "Host-
1275 Symbiont Co-Speciation and Reductive Genome Evolution in Gut Symbiotic Bacteria of
1276 Acanthosomatid Stinkbugs." *BMC Biology* 7.
1277 Klindworth, Anna, Elmar Pruesse, Timmy Schweer, et al. 2012. "Evaluation of General 16S
1278 Ribosomal RNA Gene PCR Primers for Classical and Next-Generation Sequencing-
1279 Based Diversity Studies." *Nucleic Acids Research* 41 (1): e1–e1.
1280 https://doi.org/10.1093/nar/gks808.
1281 Koch, Hauke, Dharam P. Abrol, Jilian Li, and Paul Schmid-Hempel. 2013. "Diversity and
1282 Evolutionary Patterns of Bacterial Gut Associates of Corbiculate Bees." *Molecular*
1283 *Ecology* 22 (7): 2028–44. https://doi.org/10.1111/mec.12209.
1284 Kwong, W. K., and N. A. Moran. 2015. "Evolution of Host Specialization in Gut Microbes: The
1285 Bee Gut as a Model." *Gut Microbes* 6.
1286 Lahti, Leo, and Sudarshan Shetty. 2017. *Tools for Microbiome Analysis in R Version 1.26.0*. V.
1287 1.26.0. Released. http://microbiome.github.com/microbiome.
1288 Lenth, Russell V. 2022. *Emmeans: Estimated Marginal Means, Aka Least-Squares Means*. V. R
1289 package version 1.7.2. Released. https://CRAN.R-project.org/package=emmeans.

1290 Letunic, Ivica, and Peer Bork. 2016. "Interactive Tree of Life (iTOL) v3: An Online Tool for the
1291 Display and Annotation of Phylogenetic and Other Trees." *Nucleic Acids Research* 44
1292 (W1): W1.
1293 Lewis, Sara M., Lynn Faust, and Raphaël De Cock. 2011. "The Dark Side of the Light Show:
1294 Predators of Fireflies in the Great Smoky Mountains." *Psyche* 2012 (December): 634027.
1295 https://doi.org/10.1155/2012/634027.
1296 Lewis, Sara M, Choong Hay Wong, Avalon C S Owens, et al. 2020. "A Global Perspective on
1297 Firefly Extinction Threats." *BioScience* 70 (2): 2. https://doi.org/10.1093/biosci/biz157.
1298 Ley, R. E., M. Hamady, C. Lozupone, et al. 2008. "Evolution of Mammals and Their Gut
1299 Microbes." *Science* 320.
1300 Liu, Bing-Qi, Xi-Yu Bao, Jin-Yang Yan, et al. 2024. "*Rickettsia* Symbionts Spread via Mixed
1301 Mode Transmission, Increasing Female Fecundity and Sex Ratio Shift by Host Hormone
1302 Modulating." *Proceedings of the National Academy of Sciences* 121 (25): e2406788121.
1303 https://doi.org/10.1073/pnas.2406788121.
1304 Lloyd, James E. 1965. "Aggressive Mimicry in Photuris: Firefly Femmes Fatales." *Science* (US)
1305 149 (Whole No. 3684): Whole No. 3684. https://doi.org/10.1126/science.149.3684.653.
1306 Lloyd, James E. 1969. "Flashes, Behavior and Additional Species of Nearctic Photinus Fireflies
1307 (Coleoptera: Lampyridae)." *The Coleopterists Bulletin* 23 (2): 29–40. JSTOR.
1308 Lloyd, James E. 1998. "On Research and Entomological Education II: A Conditional Mating
1309 Strategy and Resource-Sustained Lek(?) In a Classroom Firefly (Coleoptera:
1310 Lampyridae; Photinus)." *The Florida Entomologist* 81 (3): 3. JSTOR.
1311 https://doi.org/10.2307/3495917.

Lloyd, James E. 2005. “Fireflies (Coleoptera: Lampyridae).” In *Encyclopedia of Entomology*. Springer Netherlands. https://doi.org/10.1007/0-306-48380-7_1625.

Lloyd, James E. 2018. *A Naturalist’s Long Walk among Shadows: Of North American Photuris – Patterns, Outlines, Silhouettes ... Echoes.* Self-published.

Lo, Nathan, Claudio Bandi, Hirofumi Watanabe, Christine Nalepa, and Tiziana Beninati. 2003. “Evidence for Cocladogenesis Between Diverse Dictyopteran Lineages and Their Intracellular Endosymbionts.” *Molecular Biology and Evolution* 20 (6): 907–13. https://doi.org/10.1093/molbev/msg097.

Lo, Wen-Sui, Gail E Gasparich, and Chih-Horng Kuo. 2018. “Convergent Evolution among Ruminant-Pathogenic Mycoplasma Involved Extensive Gene Content Changes.” *Genome Biology and Evolution* 10 (8): 2130–39. https://doi.org/10.1093/gbe/evy172.

Lower, Sarah E., Owais Gilani, Madden J. Tuffy, Deeshani N. Patel, Zhengkai Zhu, and Moria C. Chambers. 2023. “Host Condition and Pathogen Identity Influence Bacterial Infection Survival in the Common Eastern Firefly, Photinus Pyralis.” *Ecological Entomology* 48 (1): 1. https://doi.org/10.1111/een.13204.

Lower, Sarah Sander, J. Spencer Johnston, Kathrin F. Stanger-Hall, et al. 2017. “Genome Size in North American Fireflies: Substantial Variation Likely Driven by Neutral Processes.” *Genome Biology and Evolution* 9 (6): 6. https://doi.org/10.1093/gbe/evx097.

Lüdecke, Daniel, Mattan S. Ben-Shachar, Indrajeet Patil, Philip Waggoner, and Dominique Makowski. 2021. “Performance: An R Package for Assessment, Comparison and Testing of Statistical Models.” *Journal of Open Source Software* 6 (60): 3139. https://doi.org/10.21105/joss.03139.

1334 Mainali, Kumar P., and Eric Slud. 2022. "CooccurrenceAffinity: An R Package for Computing a
1335 Novel Metric of Affinity in Co-Occurrence Data That Corrects for Pervasive Errors in
1336 Traditional Indices." *bioRxiv*, ahead of print. https://doi.org/10.1101/2022.11.01.514801.
1337 Malard, Lucie A, Muhammad Z Anwar, Carsten S Jacobsen, and David A Pearce. 2019.
1338 "Biogeographical Patterns in Soil Bacterial Communities across the Arctic Region."
1339 *FEMS Microbiology Ecology* 95 (9): fiz128. https://doi.org/10.1093/femsec/fiz128.
1340 Mallick, Himel, Ali Rahnavard, Lauren J. McIver, et al. 2021. "Multivariable Association
1341 Discovery in Population-Scale Meta-Omics Studies." *PLoS Computational Biology* 17
1342 (11): 1–27. https://doi.org/10.1371/journal.pcbi.1009442.
1343 Manthey, Christin, Paul R. Johnston, Shinichi Nakagawa, and Jens Rolff. 2023. "Complete
1344 Metamorphosis and Microbiota Turnover in Insects." *Molecular Ecology* 32 (23): 6543–
1345 51. https://doi.org/10.1111/mec.16673.
1346 Martin, Gavin J., Marc A. Branham, Michael F. Whiting, and Seth M. Bybee. 2017. "Total
1347 Evidence Phylogeny and the Evolution of Adult Bioluminescence in Fireflies
1348 (Coleoptera: Lampyridae)." *Molecular Phylogenetics and Evolution* 107: 564–75.
1349 https://doi.org/10.1016/j.ympev.2016.12.017.
1350 Martin, Gavin J, Kathrin F Stanger-Hall, Marc A Branham, et al. 2019. "Higher-Level
1351 Phylogeny and Reclassification of Lampyridae (Coleoptera: Elateroidea)." *Insect
1352 Systematics and Diversity* 3 (6): 6. https://doi.org/10.1093/isd/ixz024.
1353 Martin, Marcel. 2011. "Cutadapt Removes Adapter Sequences from High-Throughput
1354 Sequencing Reads." *EMBnet.Journal* 17 (1): 10–12.

1355 Martin, Owen, Chantal Nguyen, Raphael Sarfati, et al. 2024. "Embracing Firefly Flash Pattern
1356 Variability with Data-Driven Species Classification." *Scientific Reports* 14 (1): 3432.
1357 https://doi.org/10.1038/s41598-024-53671-3.
1358 Martinez, Arbizu, P. 2020. *pairwiseAdonis: Pairwise Multilevel Comparison Using Adonis. R*
1359 *Package Version 0.4.1.* V. 0.4.1. Released.
1360 https://github.com/pmartinezarbizu/pairwiseAdonis.
1361 Martiny, Jennifer B. Hughes, Brendan J.M. Bohannan, James H. Brown, et al. 2006. "Microbial
1362 Biogeography: Putting Microorganisms on the Map." *Nature Reviews Microbiology* 4
1363 (2): 102–12. https://doi.org/10.1038/nrmicro1341.
1364 Mazel, Florent, Katherine M. Davis, Andrew Loudon, Waldan K. Kwong, Mathieu Groussin,
1365 and Laura Wegener Parfrey. 2018. "Is Host Filtering the Main Driver of Phylosymbiosis
1366 across the Tree of Life?" *mSystems* 3 (5): 10.1128/msystems.00097-18.
1367 https://doi.org/10.1128/msystems.00097-18.
1368 McCutcheon, John P., Bradon R. McDonald, and Nancy A. Moran. 2009. "Convergent Evolution
1369 of Metabolic Roles in Bacterial Co-Symbionts of Insects." *Proceedings of the National*
1370 *Academy of Sciences* 106 (36): 36. https://doi.org/10.1073/pnas.0906424106.
1371 McDaniel, Melissa S., Natalie R. Lindgren, Caitlin E. Billiot, Kristina N. Valladares, Nicholas
1372 A. Sumpter, and W. Edward Swords. 2023. "Pseudomonas Aeruginosa Promotes
1373 Persistence of Stenotrophomonas Maltophilia via Increased Adherence to Depolarized
1374 Respiratory Epithelium." *Microbiology Spectrum* 11 (1): e03846-22.
1375 https://doi.org/10.1128/spectrum.03846-22.
1376 McDaniel, Melissa S., Trenton Schoeb, and W. Edward Swords. 2020. "Cooperativity between
1377 Stenotrophomonas Maltophilia and Pseudomonas Aeruginosa during Polymicrobial

Airway Infections." *Infection and Immunity* 88 (4): 10.1128/iai.00855-19. https://doi.org/10.1128/iai.00855-19.

McDermott, Frank A. 1967. "The North American Fireflies of the Genus Photuris Dejean a Modification of Barber's Key (Coleoptera; Lampyridae)." *The Coleopterists Bulletin* 21 (4): 106–16. JSTOR.

McFall-Ngai, Margaret, Michael G. Hadfield, Thomas C. G. Bosch, et al. 2013. "Animals in a Bacterial World, a New Imperative for the Life Sciences." *Proceedings of the National Academy of Sciences* 110 (9): 9. https://doi.org/10.1073/pnas.1218525110.

McKenna, Duane D., Seunggwan Shin, Dirk Ahrens, et al. 2019. "The Evolution and Genomic Basis of Beetle Diversity." *Proceedings of the National Academy of Sciences* 116 (49): 49. https://doi.org/10.1073/pnas.1909655116.

McLaren, M. 2020. *Speedyseq: Faster Implementations of Common Phyloseq Functions. R Package Version 0.5.3.9021*. https://github.com/mikemc/speedyseq.

McLean, Miriam, John Buck, and Frank E. Hanson. 1972. "Culture and Larval Behavior of Photurid Fireflies." *The American Midland Naturalist* 87 (1): 1. JSTOR. https://doi.org/10.2307/2423887.

McMurdie, Paul J., and Susan Holmes. 2013. "Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data." *PLOS ONE* 8 (4): e61217. https://doi.org/10.1371/journal.pone.0061217.

McMurdie, Paul J., and Susan Holmes. 2014. "Waste Not, Want Not: Why Rarefying Microbiome Data Is Inadmissible." *PLOS Computational Biology* 10 (4): e1003531. https://doi.org/10.1371/journal.pcbi.1003531.

Meadow, James F., Ashley C. Bateman, Keith M. Herkert, Timothy K. O'Connor, and Jessica L. Green. 2013. "Significant Changes in the Skin Microbiome Mediated by the Sport of Roller Derby." *PeerJ* 1 (March): e53. https://doi.org/10.7717/peerj.53.

Michalik, Anna, and Teresa Szklarzewicz. 2025. "Together Forever: Patterns and Strategies of Vertical Symbiont Transmission in Planthoppers." *Developmental Biology* 525: 319–30. https://doi.org/10.1016/j.ydbio.2025.06.020.

Miller, Mark A, Wayne Pfeiffer, and Terri Schwartz. 2010. "Creating the CIPRES Science Gateway for Inference of Large Phylogenetic Trees." 1–8.

Moran, Nancy A., and Helen E. Dunbar. 2006. "Sexual Acquisition of Beneficial Symbionts in Aphids." *Proceedings of the National Academy of Sciences* 103 (34): 12803–6. https://doi.org/10.1073/pnas.0605772103.

Ning, Daliang, Ye Deng, James M. Tiedje, and Jizhong Zhou. 2019. "A General Framework for Quantitatively Assessing Ecological Stochasticity." *Proceedings of the National Academy of Sciences* 116 (34): 16892–98. https://doi.org/10.1073/pnas.1904623116.

Oba, Y., K. Konishi, D. Yano, H. Shibata, D. Kato, and T. Shirai. 2020. "Resurrecting the Ancient Glow of the Fireflies." *Science Advances* 6 (49): 49. https://doi.org/10.1126/sciadv.abc5705.

Ohbayashi, Tsubasa, Kazutaka Takeshita, Wataru Kitagawa, et al. 2015. "Insect's Intestinal Organ for Symbiont Sorting." *Proceedings of the National Academy of Sciences* 112 (37): 37. https://doi.org/10.1073/pnas.1511454112.

Oksanen, Jari, F Guillaume Blanchet, Michael Friendly, et al. 2016. "Vegan: Community Ecology Package. R Package Version 2.4-3." *Vienna: R Foundation for Statistical Computing*.

1423 Oliphant, Kaitlyn, Valeria R Parreira, Kyla Cochrane, and Emma Allen-Vercoe. 2019. "Drivers
1424 of Human Gut Microbial Community Assembly: Coadaptation, Determinism and
1425 Stochasticity." *The ISME Journal* 13 (12): 3080–92. https://doi.org/10.1038/s41396-019-
1426 0498-5.
1427 Othman, Nurul, Nur Abdullah, Roslim Ramli, Nur Khairunnisa Salleh, and Norela Sulaiman.
1428 2018. "Mouthpart and Digestive Tract Morphology of the Synchronized Firefly,
1429 Pteroptyx Tener (Coleoptera: Lampyridae)." *Serangga* 23 (2): 2.
1430 Parada, Alma E., David M. Needham, and Jed A. Fuhrman. 2016. "Every Base Matters:
1431 Assessing Small Subunit rRNA Primers for Marine Microbiomes with Mock
1432 Communities, Time Series and Global Field Samples." *Environmental Microbiology* 18
1433 (5): 1403–14. https://doi.org/10.1111/1462-2920.13023.
1434 Paradis, E., J. Claude, and K. Strimmer. 2004. "APE: Analyses of Phylogenetics and Evolution
1435 in R Language." *Bioinformatics* 20.
1436 Pebesma, Edzer. 2018. "Simple Features for R: Standardized Support for Spatial Vector Data."
1437 *The R Journal* 10 (1): 439–46. https://doi.org/10.32614/RJ-2018-009.
1438 Perlmutter, Jessamyn I., and Seth R. Bordenstein. 2020. "Microorganisms in the Reproductive
1439 Tissues of Arthropods." *Nature Reviews Microbiology* 18 (2): 97–111.
1440 https://doi.org/10.1038/s41579-019-0309-z.
1441 Perreau, Julie, and Nancy A. Moran. 2022. "Genetic Innovations in Animal–Microbe
1442 Symbioses." *Nature Reviews Genetics* 23 (1): 23–39. https://doi.org/10.1038/s41576-
1443 021-00395-z.
1444 Pinheiro, José, Douglas Bates, and R Core Team. 2024. *Nlme: Linear and Nonlinear Mixed*
1445 *Effects Models*. https://CRAN.R-project.org/package=nlme.

Powell, Gareth S., Natalie A. Saxton, Yelena M. Pacheco, et al. 2022. "Beetle Bioluminescence Outshines Extant Aerial Predators." *Proceedings of the Royal Society B: Biological Sciences* 289 (1979): 1979. https://doi.org/10.1098/rspb.2022.0821.

Quast, Christian, Elmar Pruesse, Pelin Yilmaz, et al. 2013. "The SILVA Ribosomal RNA Gene Database Project: Improved Data Processing and Web-Based Tools." *Nucleic Acids Research* 41 (D1): D590–96. https://doi.org/10.1093/nar/gks1219.

R Core Team. 2024. *R: A Language and Environment for Statistical Computing.* R. R Foundation for Statistical Computing. R Foundation for Statistical Computing, released. https://www.R-project.org/.

Ravenscraft, Alison, Michelle Berry, Tobin Hammer, Kabir Peay, and Carol Boggs. 2019. "Structure and Function of the Bacterial and Fungal Gut Microbiota of Neotropical Butterflies." *Ecological Monographs* 89 (2): 2. https://doi.org/10.1002/ecm.1346.

Ravenscraft, Alison, Suzanne Kelly, David Haviland, Johnathan Adamson, and Molly Hunter. 2024. "Spatial and Temporal Structure of Environmentally-Acquired Caballeronia Symbionts of a Leaffooted Bug." *Authorea*, ahead of print, September. https://doi.org/10.22541/au.172518956.62905743/v1.

Rennstam Rubbmark, Oskar, Daniela Sint, Nina Horngacher, and Michael Traugott. 2018. "A Broadly Applicable COI Primer Pair and an Efficient Single-Tube Amplicon Library Preparation Protocol for Metabarcoding." *Ecology and Evolution* 8 (24): 12335–50. https://doi.org/10.1002/ece3.4520.

Riley, William B., Simone Policena Rosa, and Luiz Felipe Lima da Silveira. 2021. "A Comprehensive Review and Call for Studies on Firefly Larvae." *PeerJ* 9 (September): e12121. https://doi.org/10.7717/peerj.12121.

Rooney, Jennifer A., and Sara M. Lewis. 2000. “Notes on the Life History and Mating Behavior of Ellychnia Corrusca (Coloeptera: Lampyridae).” *The Florida Entomologist* 83 (3): 3. JSTOR. https://doi.org/10.2307/3496351.

Russell, Jacob A., Stephanie Weldon, Andrew H. Smith, et al. 2013. “Uncovering Symbiont-Driven Genetic Diversity across North American Pea Aphids.” *Molecular Ecology* 22 (7): 2045–59. https://doi.org/10.1111/mec.12211.

Sacchi, L, C A Nalepa, M Lenz, et al. 2000. “Transovarial Transmission of Symbiotic Bacteria in Mastotermes Darwiniensis (Isoptera: Mastotermitidae): Ultrastructural Aspects and Phylogenetic Implications.” *Annals of the Entomological Society of America* 93 (6): 1308–13. https://doi.org/10.1603/0013-8746(2000)093%255B1308:TTOSBI%255D2.0.CO;2.

Sachs, Joel L, Ulrich G Mueller, Thomas P Wilcox, and James J Bull. 2004. “The Evolution of Cooperation.” *The Quarterly Review of Biology* 79 (2): 135–60. https://doi.org/10.1086/383541.

Sander, S. E., and D. W. Hall. 2015. “Variation in Opsin Genes Correlates with Signalling Ecology in North American Fireflies.” *Molecular Ecology* 24 (18): 4679–96. https://doi.org/10.1111/mec.13346.

Schloss, Patrick D. 2023. “Waste Not, Want Not: Revisiting the Analysis That Called into Question the Practice of Rarefaction.” *mSphere* 9 (1): e00355-23. https://doi.org/10.1128/msphere.00355-23.

Schloss, Patrick D. 2024. “Rarefaction Is Currently the Best Approach to Control for Uneven Sequencing Effort in Amplicon Sequence Analyses.” *mSphere* 9 (2): e00354-23. https://doi.org/10.1128/msphere.00354-23.

1492 Searle, S. R., F. M. Speed, and G. A. Milliken. 1980. "Population Marginal Means in the Linear
1493 Model: An Alternative to Least Squares Means." *The American Statistician* 34 (4): 4.
1494 https://doi.org/10.1080/00031305.1980.10483031.
1495 Sievers, F., A. Wilm, D. Dineen, et al. 2011. "Fast, Scalable Generation of High-Quality Protein
1496 Multiple Sequence Alignments Using Clustal Omega." *Mol Syst Biol* 7.
1497 Simon, Chris, Francesco Frati, Andrew Beckenbach, Bernie Crespi, Hong Liu, and Paul Flook.
1498 1994. "Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences
1499 and a Compilation of Conserved Polymerase Chain Reaction Primers." *Annals of the*
1500 *Entomological Society of America* 87 (6): 651–701.
1501 https://doi.org/10.1093/aesa/87.6.651.
1502 Sloan, William T., Mary Lunn, Stephen Woodcock, Ian M. Head, Sean Nee, and Thomas P.
1503 Curtis. 2006. "Quantifying the Roles of Immigration and Chance in Shaping Prokaryote
1504 Community Structure." *Environmental Microbiology* 8 (4): 732–40.
1505 https://doi.org/10.1111/j.1462-2920.2005.00956.x.
1506 Smedley, Scott R., Riley G. Risteen, Kathareeya K. Tonyai, et al. 2017. "Bufadienolides
1507 (Lucibufagins) from an Ecologically Aberrant Firefly (Ellychnia Corrusca)."
1508 *Chemoecology* 27 (4): 141–53. https://doi.org/10.1007/s00049-017-0240-6.
1509 Stamatakis, Alexandros. 2014. "RAxML Version 8: A Tool for Phylogenetic Analysis and Post-
1510 Analysis of Large Phylogenies." *Bioinformatics* 30 (9): 9.
1511 Stanger-Hall, Kathrin F., and James E. Lloyd. 2015. "Flash Signal Evolution in Photinus
1512 Fireflies: Character Displacement and Signal Exploitation in a Visual Communication
1513 System." *Evolution* 69 (3): 666–82. https://doi.org/10.1111/evo.12606.

1514 Stanger-Hall, Kathrin F., James E. Lloyd, and David M. Hillis. 2007. "Phylogeny of North
1515 American Fireflies (Coleoptera: Lampyridae): Implications for the Evolution of Light
1516 Signals." *Molecular Phylogenetics and Evolution* 45 (1): 33–49.
1517 https://doi.org/10.1016/j.ympev.2007.05.013.
1518 Stevens, Clauzell, Ah Yin Tang, Edward Jenkins, et al. 1997. "Spiroplasma Lampyridicola Sp.
1519 Nov., from the Firefly Beetle Photuris Pennsylvanicus." In *International Journal of*
1520 *Systematic and Evolutionary Microbiology*, vol. 47. no. 3. Microbiology Society.
1521 https://doi.org/10.1099/00207713-47-3-709.
1522 Sudakaran, Sailendharan, Christian Kost, and Martin Kaltenpoth. 2017. "Symbiont Acquisition
1523 and Replacement as a Source of Ecological Innovation." *Trends in Microbiology* 25 (5):
1524 5. https://doi.org/10.1016/j.tim.2017.02.014.
1525 Torsvik, V., J. Goksøyr, and F. L. Daae. 1990. "High Diversity in DNA of Soil Bacteria."
1526 *Applied and Environmental Microbiology* 56 (3): 782–87.
1527 https://doi.org/10.1128/aem.56.3.782-787.1990.
1528 Tully, J. G., D. L. Rose, C. E. Yunker, et al. 1995. "Spiroplasma Ixodetis Sp. Nov., a New
1529 Species from Ixodes Pacificus Ticks Collected in Oregon." *International Journal of*
1530 *Systematic Bacteriology* (England) 45 (1): 23–28. https://doi.org/10.1099/00207713-45-
1531 1-23.
1532 Tully, JOSEPH G., DAVID L. Rose, KEVIN J. Hackett, et al. 1989. "Mycoplasma Ellychniae
1533 Sp. Nov., a Sterol-Requiring Mollicute from the Firefly Beetle Ellychnia Corrusca." In
1534 *Int J Syst Evol Microbiol*, No. 3, vol. 39. no. 3. Microbiology Society.
1535 https://doi.org/10.1099/00207713-39-3-284.

Tully, JOSEPH G., ROBERT F. Whitcomb, KEVIN J. Hackett, et al. 1994. "Taxonomic Descriptions of Eight New Non-Sterol-Requiring Mollicutes Assigned to the Genus Mesoplasma." In *Int J Syst Evol Microbiol*, No. 4, vol. 44. no. 4. Microbiology Society. https://doi.org/10.1099/00207713-44-4-685.

Tully, JOSEPH G., ROBERT F. Whitcomb, KEVIN J. Hackett, et al. 1998. "Entomoplasma Freundtii Sp. Nov., a New Species from a Green Tiger Beetle (Coleoptera: Cicindelidae)." In *Int J Syst Evol Microbiol*, No. 4, vol. 48. no. 4. Microbiology Society. https://doi.org/10.1099/00207713-48-4-1197.

Vaz, Stephanie, Maria Cecília Guerrazzi, Marizete Rocha, et al. 2021. "On the Intertidal Firefly Genus Micronaspis Green, 1948, with a New Species and a Phylogeny of Cratomorphini Based on Adult and Larval Traits (Coleoptera: Lampyridae)." *Zoologischer Anzeiger* 292: 64–91. https://doi.org/10.1016/j.jcz.2021.01.002.

Vaz, Stephanie, Luiz Felipe Lima da Silveira, and Simone Policena Rosa. 2020. "Morphology and Life Cycle of a New Species of Psilocladus Blanchard, 1846 (Coleoptera, Lampyridae, Psilocladinae), the First Known Bromeliad-Inhabiting Firefly." Original Article. *Papéis Avulsos de Zoologia* 60 (special): e202060(s.i.).24. https://doi.org/10.11606/1807-0205/2020.60.special-issue.24.

Vergara, Aldair, Yara Maquitico, and Carlos Cordero. 2025. "Interspecific Courtship Between Two Endemic Fireflies." *Diversity* 17 (3). https://doi.org/10.3390/d17030188.

Waites, Ken B., and Deborah F. Talkington. 2004. "*Mycoplasma Pneumoniae* and Its Role as a Human Pathogen." *Clinical Microbiology Reviews* 17 (4): 697–728. https://doi.org/10.1128/cmr.17.4.697-728.2004.

Wang, Qiong, George M. Garrity, James M. Tiedje, and James R. Cole. 2007. "Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy." *Applied and Environmental Microbiology* 73 (16): 5261–67. https://doi.org/10.1128/AEM.00062-07.

Wedincamp, Jr., Jim, Frank E. French, Robert F. Whitcomb, and Roberta B. Henegar. 1996. "Spiroplasmas and Entomoplasmas (Procaryotae:Mollicutes) Associated with Tabanids (Diptera:Tabanidae) and Fireflies (Coleoptera:Lampyridae)." *Journal of Invertebrate Pathology* 68 (2): 2. https://doi.org/10.1006/jipa.1996.0080.

Weiss, Sophie, Zhenjiang Zech Xu, Shyamal Peddada, et al. 2017. "Normalization and Microbial Differential Abundance Strategies Depend upon Data Characteristics." *Microbiome* 5 (1): 27. https://doi.org/10.1186/s40168-017-0237-y.

Werren, J. H, L. Baldo, and M. E Clark. 2008. "Wolbachia: Master Manipulators of Invertebrate Biology." *Nature Reviews: Microbiology* 6 (10): 10.

Williamson, DAVID L., JOSEPH G. Tully, DAVID L. Rose, et al. 1990. "Mycoplasma Somnilux Sp. Nov., Mycoplasma Luminosum Sp. Nov., and Mycoplasma Lucivorax Sp. Nov., New Sterol-Requiring Mollicutes from Firefly Beetles (Coleoptera: Lampyridae)." In *Int J Syst Evol Microbiol*, No. 2, vol. 40. no. 2. Microbiology Society. https://doi.org/10.1099/00207713-40-2-160.

Wing, Steven R. 1984. "Female Monogamy and Male Competition in Photinus Collustrans (Coleoptera: Lampyridae)." *Psyche: A Journal of Entomology* 91 (1–2): 061796. https://doi.org/10.1155/1984/61796.

Wing, Steven R. 1989. “Energetic Costs of Mating in a Flightless Female Firefly,Photinus Collustrans (Coleoptera: Lampyridae).” *Journal of Insect Behavior* 2 (6): 841–47. https://doi.org/10.1007/BF01049405.

Yan, Xiao-Hua, Shen-Chian Pei, Hsi-Ching Yen, et al. 2024. “Delineating Bacterial Genera Based on Gene Content Analysis: A Case Study of the Mycoplasmatales–Entomoplasmatales Clade within the Class Mollicutes.” In *Microbial Genomics*, vol. 10. no. 11. Microbiology Society. https://doi.org/10.1099/mgen.0.001321.

Yang, Lin-Yu, Da-Rui Tang, Fu-Xin Li, Shi-Qi Luo, Cheng-Quan Cao, and Qi-Lin Zhang. 2024. “Larval Feeding Habits of Five Firefly Species Across Aquatic, Semi-Aquatic, and Terrestrial Lineages.” *Insects* 15 (12). https://doi.org/10.3390/insects15121004.

Zaragoza-Caballero, Santiago, Martín Leonel Zurita-García, and Andrés Ramírez-Ponce. 2023. “The on–off Pattern in the Evolution of the Presence of Bioluminescence in a Derived Lineage from Fireflies of Mexico (Coleoptera, Lampyridae).” *Zoologischer Anzeiger* 302: 266–83. https://doi.org/10.1016/j.jcz.2022.12.009.

Zélé, Flore, Sara Magalhães, Sonia Kéfi, and Alison B. Duncan. 2018. “Ecology and Evolution of Facilitation among Symbionts.” *Nature Communications* 9 (1): 4869. https://doi.org/10.1038/s41467-018-06779-w.

Zhang, Wenzhe, Jiali Wang, Zhi Huang, Xiaohua He, and Cong Wei. 2023. “Symbionts in *Hodgkinia*-Free Cicadas and Their Implications for Co-Evolution between Endosymbionts and Host Insects.” *Applied and Environmental Microbiology* 89 (12): e01373-23. https://doi.org/10.1128/aem.01373-23.

Zhao, Zi-Shun, Lin-Yu Yang, Fu-Xin Li, et al. 2023. "Gut Flora Alterations among Aquatic Firefly Aquatica Leii Inhabiting Various Dissolved Oxygen in Fresh Water." *iScience* 26 (10): 107809. https://doi.org/10.1016/j.isci.2023.107809.

Zheng, Bangxiao, Lantian Su, Nan Hui, et al. 2024. "Urbanisation Shapes Microbial Community Composition and Functional Attributes More so than Vegetation Type in Urban Greenspaces across Climatic Zones." *Soil Biology and Biochemistry* 191: 109352. https://doi.org/10.1016/j.soilbio.2024.109352.

Zhou, Jizhong, and Daliang Ning. 2017. "Stochastic Community Assembly: Does It Matter in Microbial Ecology?" *Microbiology and Molecular Biology Reviews* 81 (4): 10.1128/mmbr.00002-17. https://doi.org/10.1128/mmbr.00002-17.

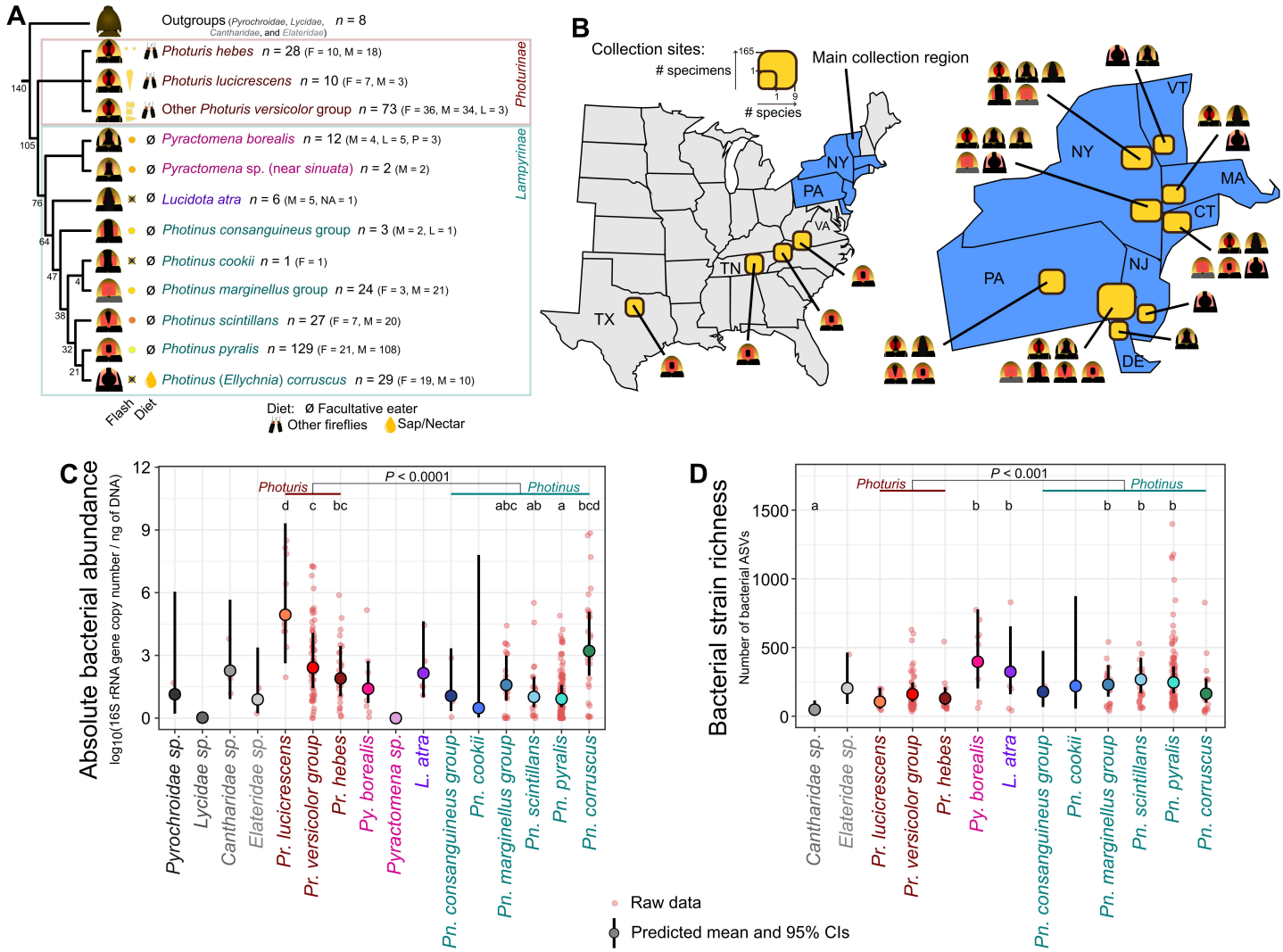

Figure 1: Sampling overview and firefly microbiome abundance and diversity across species. **A**. Species sampled and analyzed in the present study. The phylogenetic tree is adapted from Catalán et al. (2022) and Martin et al. (2019). Numbers at each node indicate estimated divergence time from Catalán 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 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. **D**. 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 S4 and Figure S4.

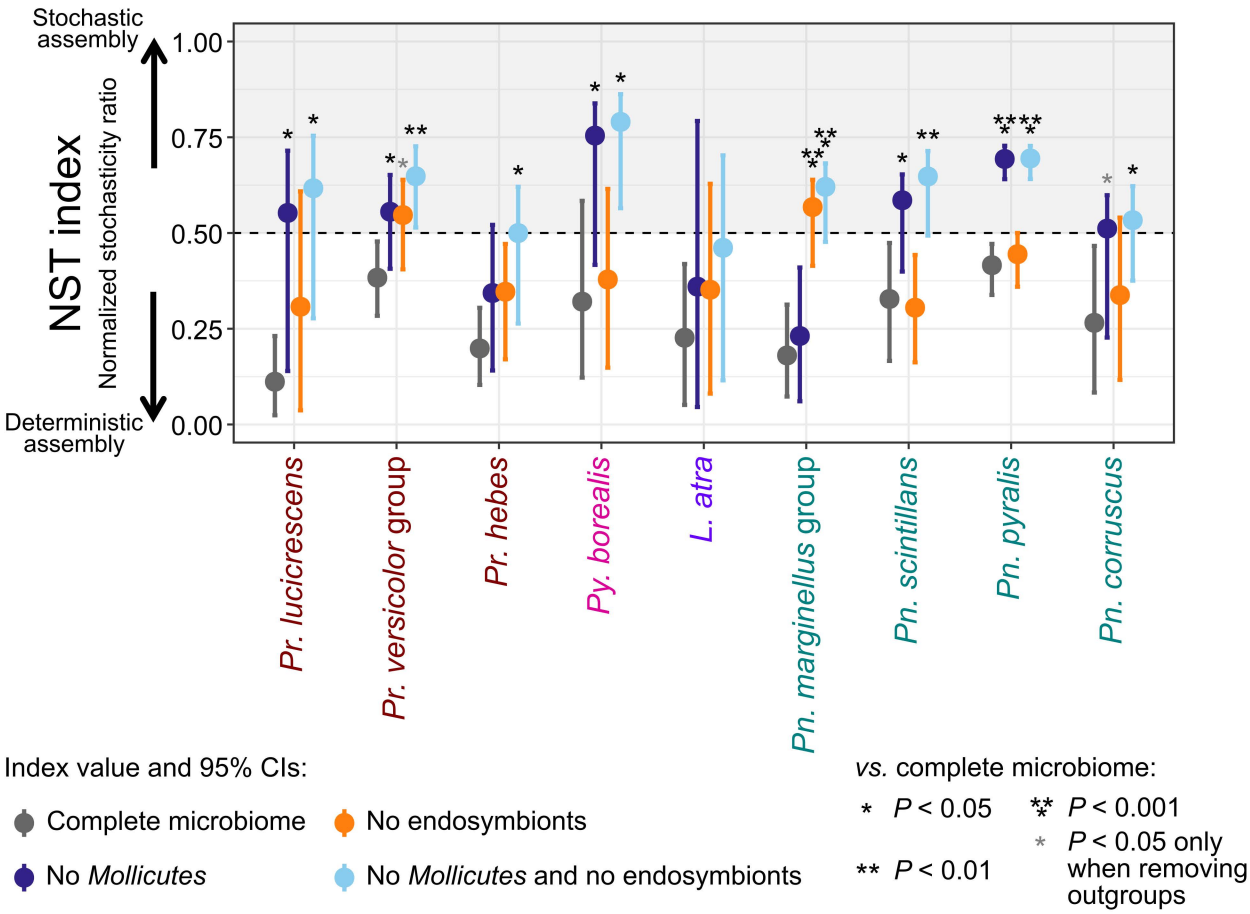

Figure 2: Normalized stochasticity ratio (NST) across firefly species. NST index values higher than 0.5 indicate a higher importance of stochastic forces governing bacterial community assembly, whereas values lower than 0.5 point to deterministic forces as the main drivers of community assembly. For 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 S2.

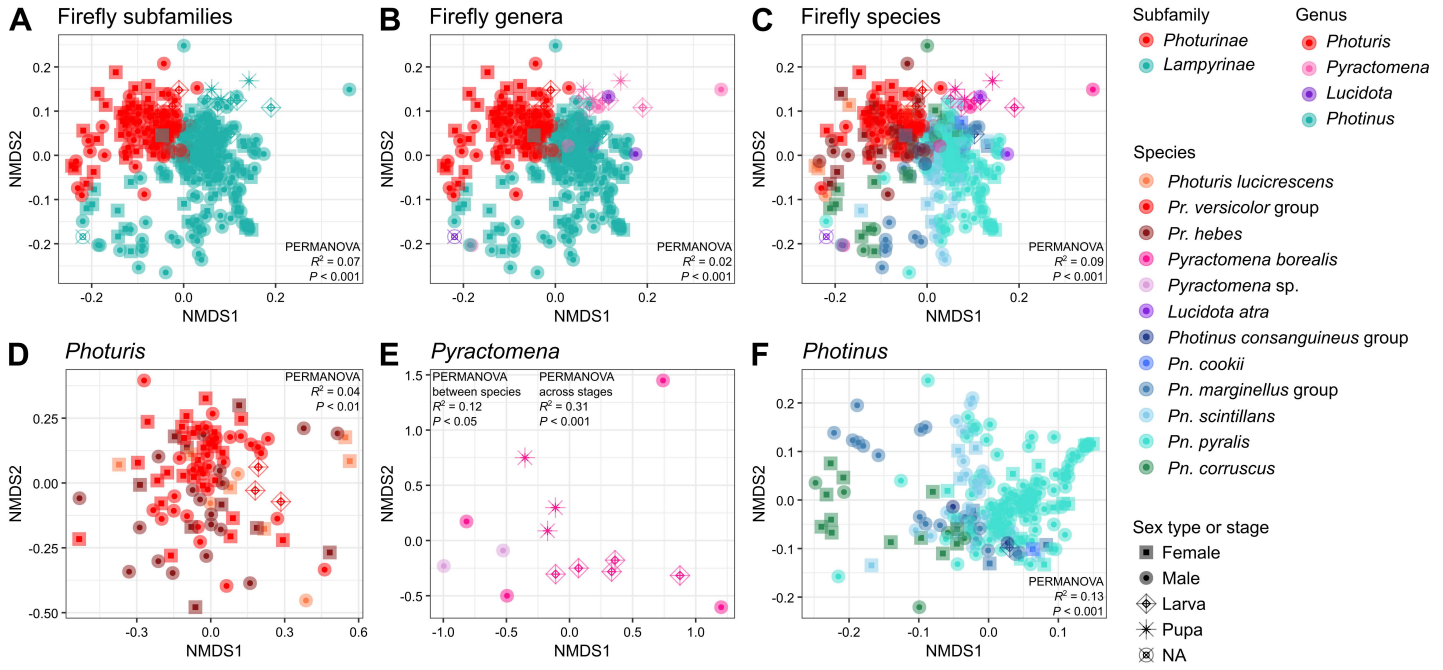

Figure 3: Microbiome divergence across fireflies at different taxonomic levels. **A**, **B** and **C**. Non-metric multidimensional scaling (NMDS) analyses showing separation of the bacterial communities based on composition and relative abundance at the firefly subfamily, genus, and species levels. 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.

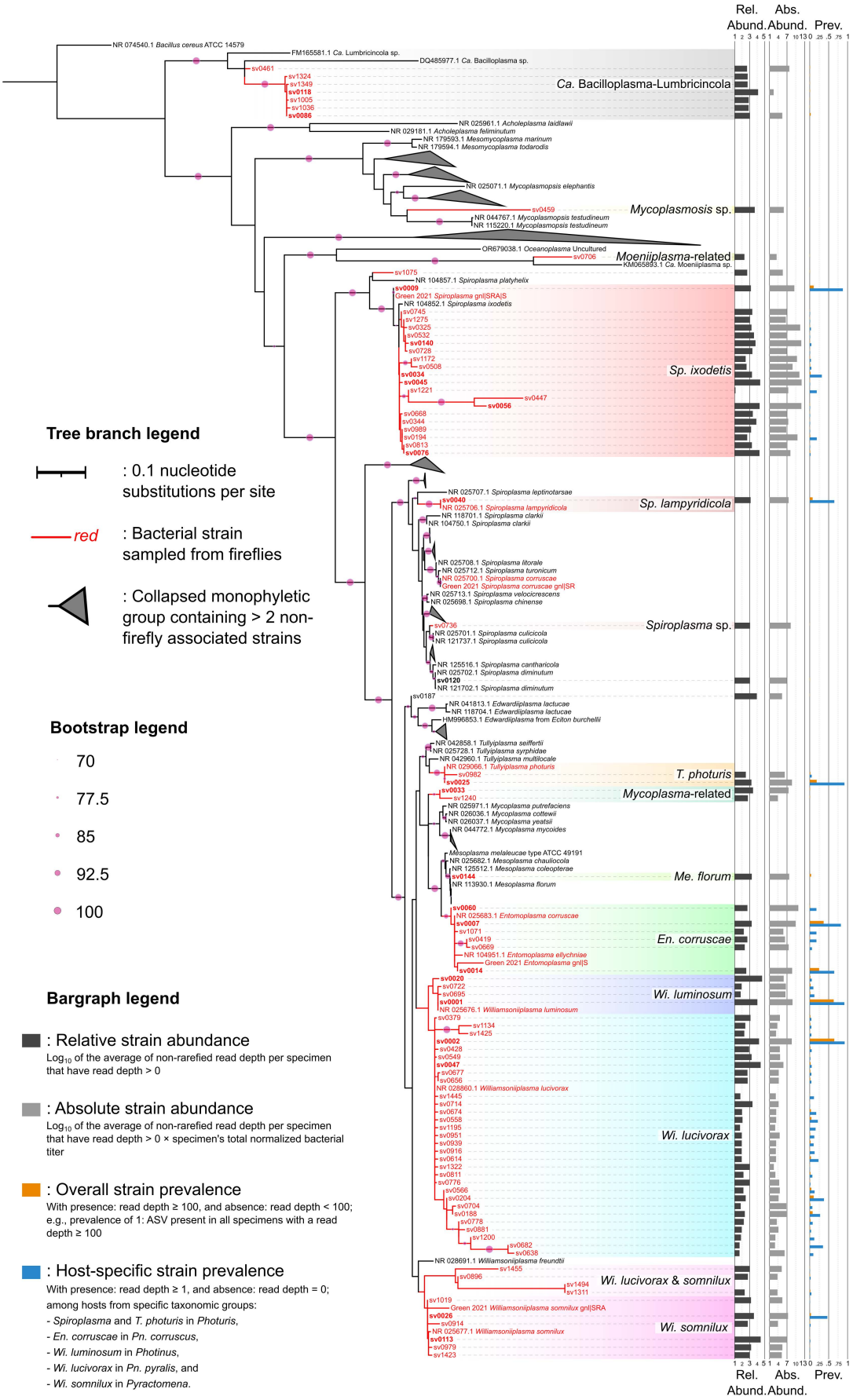

Figure 4: Phylogenetic strain diversity of the *Mollicutes* associated with fireflies. The tree was obtained from a maximum likelihood phylogenetic analysis on the 16S rRNA gene, rooted with a *Bacillus cereus* sequence (NR_074540.1), and contains 230 leaves of which 88 are 16S rRNA amplicon sequences 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 Figure S9. 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.

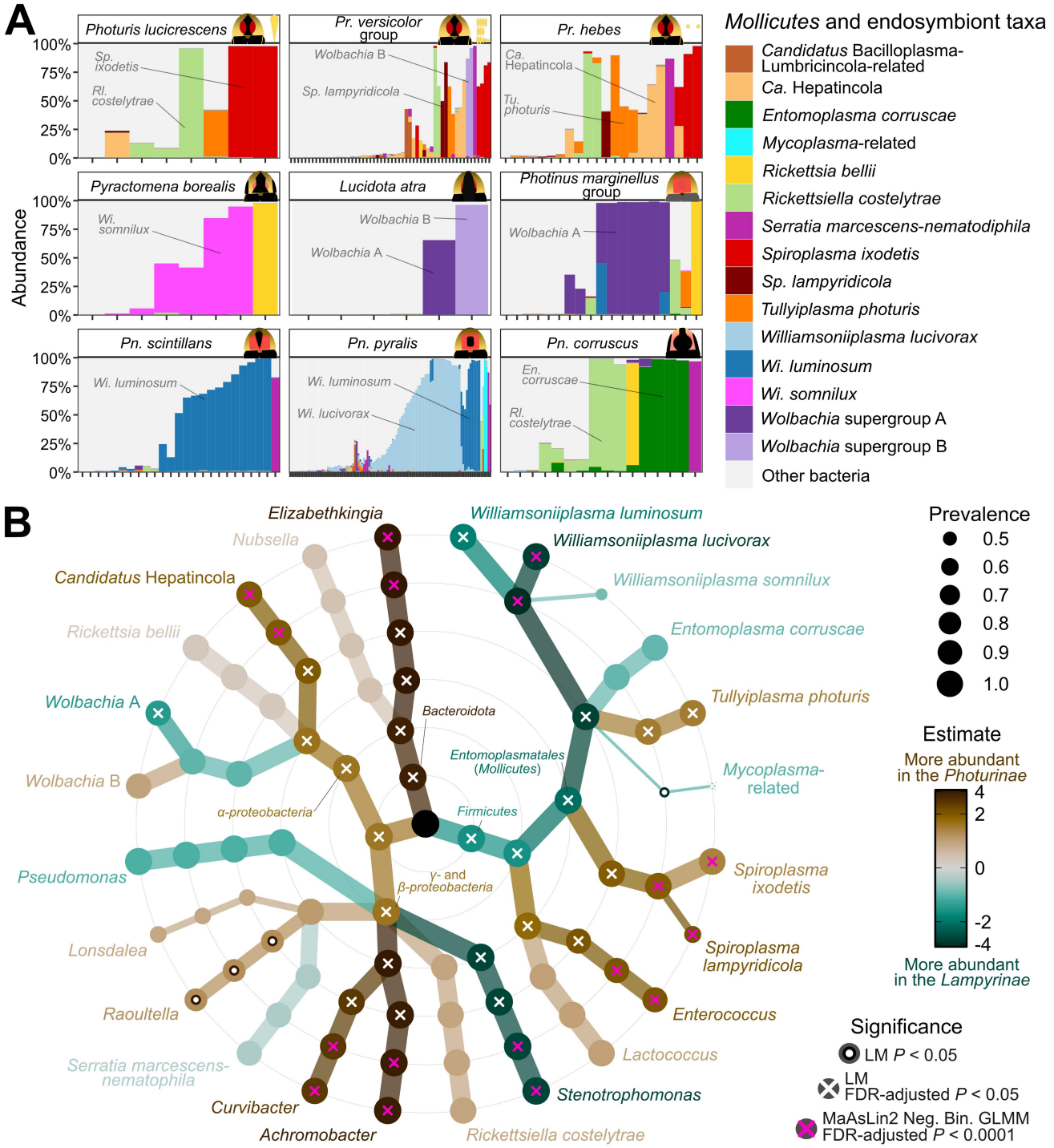

Figure 5: Bacterial composition and differential abundance. **A**. Composition of bacterial taxa across firefly species and species groups with more than three specimens in our dataset post rarefying. Each vertical bar (x-axis) represents one firefly specimen. The relative rarefied abundance (y-axis) 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 Figure S12. **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 $P < 0.001$.

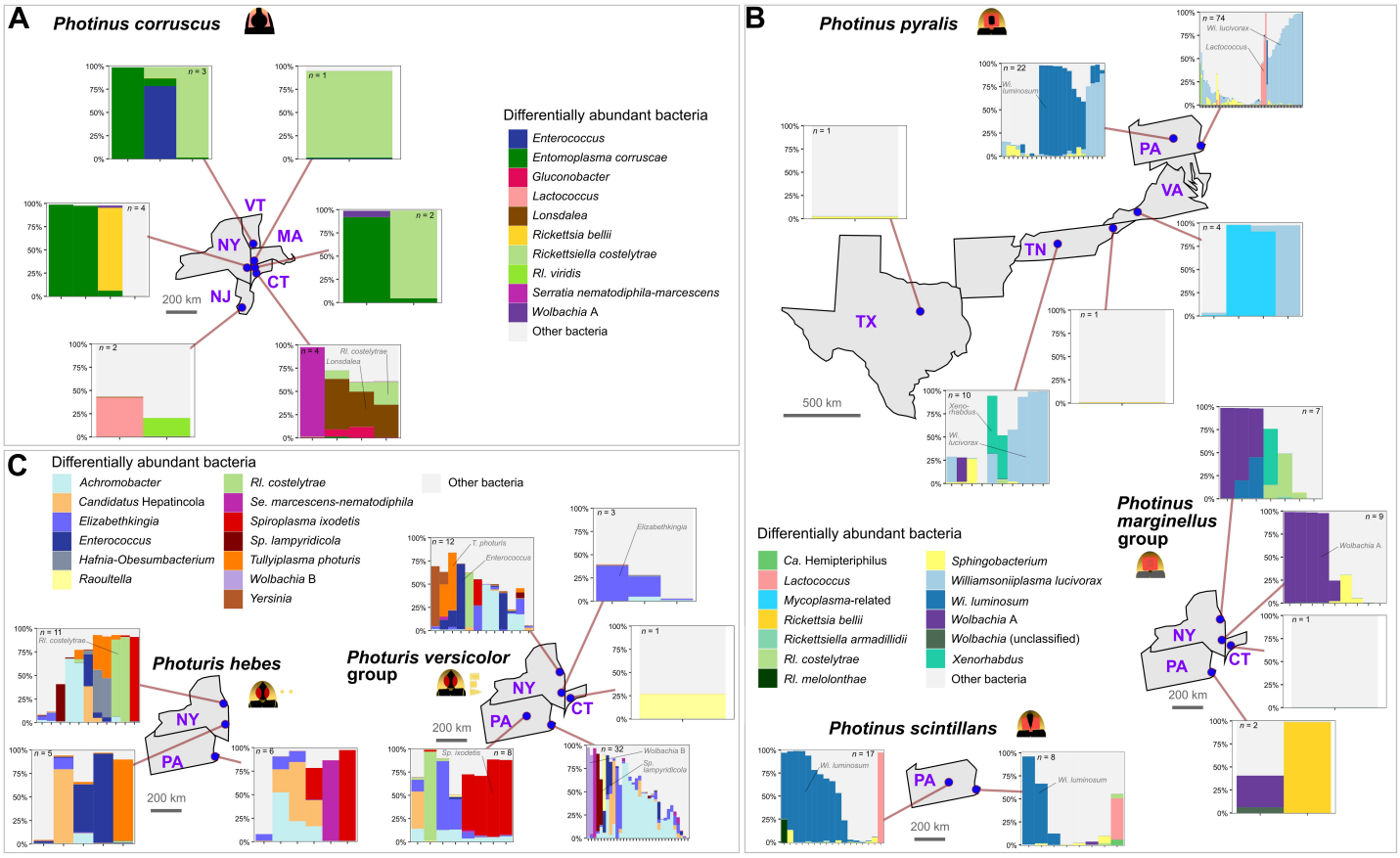

Figure 6: Regional differences in bacterial composition. Bacterial composition, with a focus on the most differentially abundant bacterial taxa across regions. Firefly species are grouped by microbiome similarity, with *Photinus corruscus* in **A**, *Pn. pyralis*, *Pn. marginellus* group, and *Pn. scintillans* 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 S6.

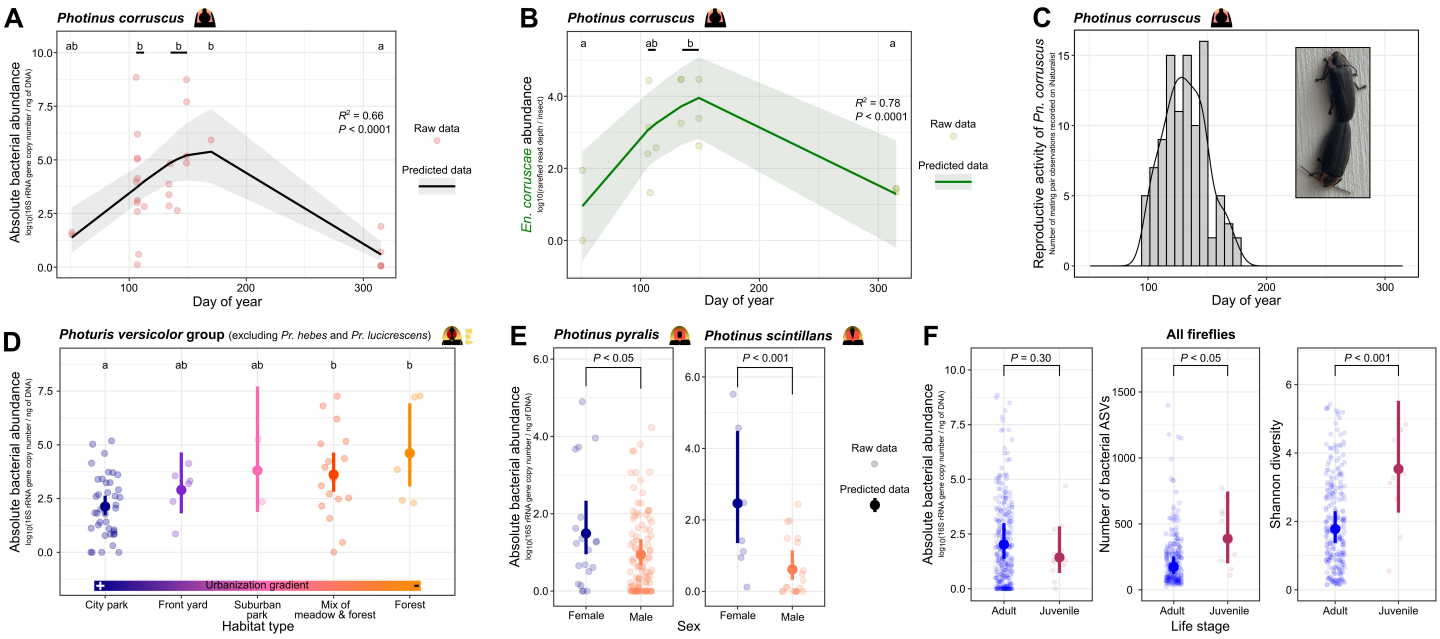

Figure 7: Seasonal, environmental, and life history factors influence the microbiome of fireflies. **A**. Normalized absolute bacterial abundance in *Photinus corruscus* peaks at the end of spring. A generalized linear mixed model (GLMM) was used to test whether bacterial titer is influenced by day of 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.

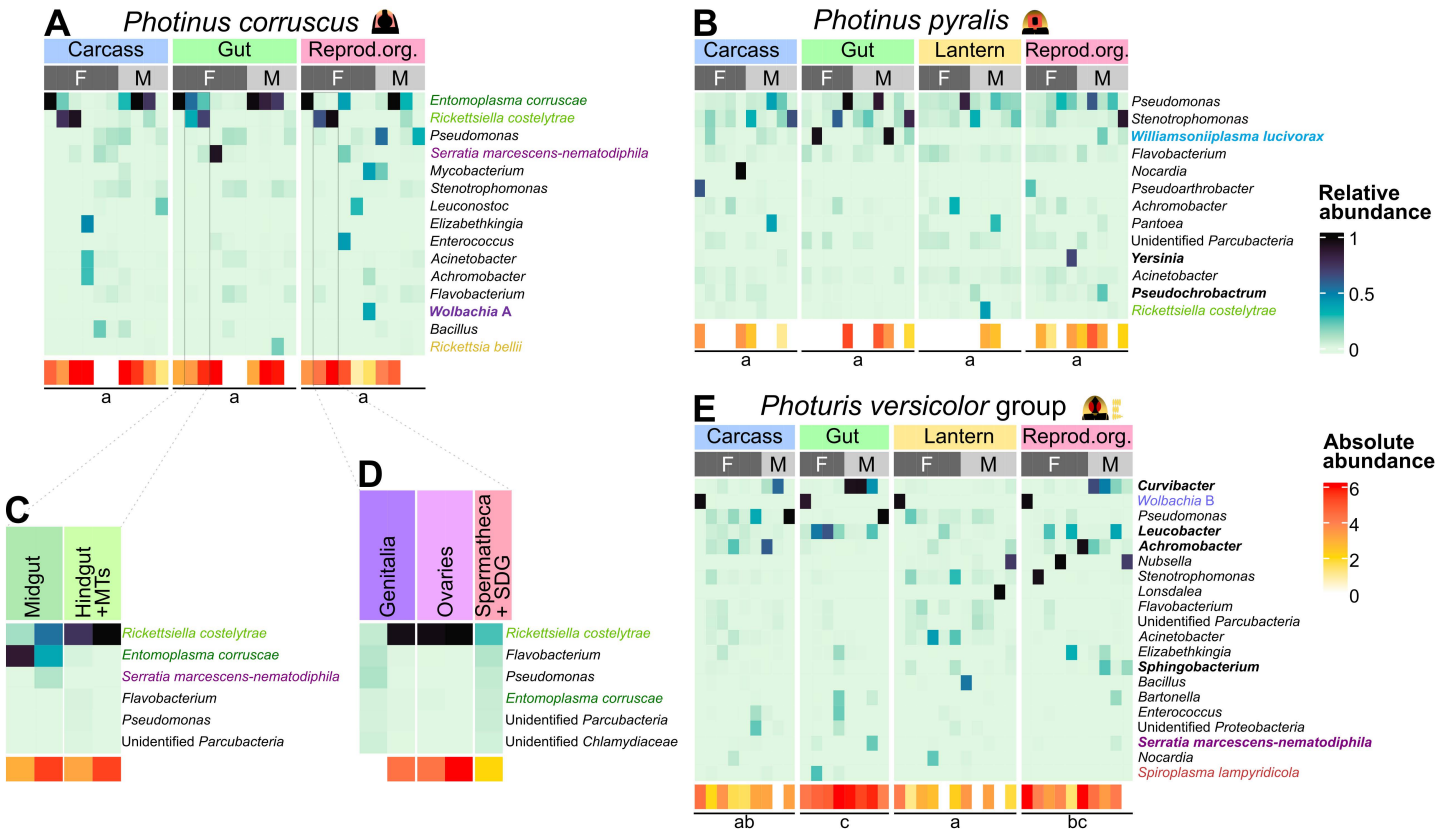

Figure 8: Microbiome changes across tissues in three firefly species. A, **B**, and **E**. Bacterial community composition and abundance across the carcass, gut, reproductive organs ("Reprod. Org."), and lantern in *Photinus corruscus*, *Pn. pyralis*, and unidentified species from the *Photuris versicolor* 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 S9. 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 Figure S22–S24, and dissection information can be found in Table S8.