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The dispersal of microbes among and within flowers by butterflies

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Abstract

1. Floral microbes, including bacteria and fungi, alter nectar quality, thus changing pollinator visitation. Conversely, pollinator visitation can change the floral microbial community.
2. Most studies on dispersal of floral microbes have focused on bees, ants or hummingbirds, yet Lepidoptera are important pollinators.
3. We asked (a) where are microbes present on the butterfly body, (b) do butterflies transfer microbes while foraging, and (c) how does butterfly foraging affect microbial abundance on different floret structures.
4. The tarsi and proboscis had significantly more microbes than the thorax in wild-caught *Glaucopsyche lygdamus* (Lepidoptera: Lycaenidae) and *Speyeria mormonia* (Lepidoptera: Nymphalidae). *Glaucopsyche lygdamus*, a smaller-bodied species, had fewer microbes than *S. mormonia*.
5. As a marker for microbes, we used a bacterium (*Rhodococcus fascians*, near NCBI Y11196) isolated from a *S. mormonia* that was foraging for nectar, and examined its dispersal by *G. lygdamus* and *S. mormonia* visiting florets of *Pyrrocoma crocea* (Asteraceae). Microbial dispersal among florets correlated positively with bacterial abundance in the donor floret. Dispersal also depended on butterfly species, age, and bacterial load carried by the butterfly.
6. Recipient florets had less bacteria than donor florets. The nectaries had more bacteria than the anthers or the stigmas, while anthers and stigmas did not differ from each other. There was no differential transmission among floral organs.
7. Lepidoptera thus act as vectors of floral microbes. Including Lepidoptera is thus crucial to an understanding of plant–pollinator–microbe interactions. Future studies should consider the role of vectored microbes in lepidopteran ecology and fitness.

KEYWORDS
Lepidoptera, microbial transmission, nectar microbes, nectaries, pollinator

INTRODUCTION

Pollinator visitation to flowers can change the composition and diversity of the floral microbial community (Ushio et al., 2015; Vannette & Fukami, 2017; Vega et al., 2021). In turn, flowers serve as nutrient-rich environments for diverse and abundant microbes (Aleklett et al., 2014). Microbial communities in flowers can change the physical and chemical environment within the flowers. Yeasts

can alter the composition and concentration of amino acids and sugars (Canto & Herrera, 2012), augment floral volatile emission (Raguso, 2004; Rering et al., 2021) and warm nectar (Herrera & Pozo, 2010). Nectar-inhabiting yeasts can have positive, indirect, pollinator-mediated effects on plant fitness and can act as a signal to attract pollinators (Schaeffer & Irwin, 2014; Yang et al., 2019). Bacteria can modify the physical and chemical traits of nectar including sugar and amino acid content and concentration (Canto & Herrera, 2012), pH (Lenaerts et al., 2016), scent (Golonka et al., 2014) and presence of secondary metabolites (Vannette & Fukami, 2016). These changes can alter nectar quality, which can in turn cause pollinators to avoid certain flowers (Good et al., 2014; Herrera et al., 2008) and weaken plant–pollinator mutualisms (Vannette et al., 2013). Hence, plant–pollinator interactions can iteratively impact the spread of microbes among and within both plants and pollinators (Graystock et al., 2015).

Among the gaps in our understanding of microbe-flower-pollinator interactions, two are examined here. First, the dispersal of floral microbes has been studied almost exclusively in the context of pollinators foraging for nectar (Herrera et al., 2008; Hausmann et al., 2017). However, flowers are complex structures comprised of many organs with varied purposes. Pollinators interact with floral organs to differing degrees. For example, bumble bees disperse microbes at different rates to different floral organs while foraging for nectar and pollen (Russell et al., 2019). Floral organs also differ in morphology, function and habitat suitability for microbes, which influence microbial communities (Aleklett et al., 2014; Junker & Keller, 2015; Steven et al., 2018).

A second gap in our knowledge is that studies of pollinators other than bees and hummingbirds are scarce (Cullen et al., 2021). Butterflies, like other pollinators, have the potential to act as vectors among plants for microbes, including bacteria and fungi, and butterfly foraging behaviour differs from that of other pollinators (e.g., Schmitt, 1980). Given that pollen has been found on the proboscis, body and legs of butterflies (Levin & Berube, 1972; Murphy, 1984), these body parts could also be instrumental in transfer of microbes. Certain butterfly morphological traits (e.g., body mass, wing loading and proboscis length) are adapted to the morphology of the flowers that the butterflies visit, which influences pollen and potentially microbe transfer efficiency (Corbet, 2000). It is, however, unknown what butterfly body parts are involved in microbe transfer, and there is a paucity of studies of the rate at which these pollinators acquire and disperse microbes among and within flowers. We ask whether butterflies acquire microbes while foraging, disperse microbes between flowers, and disperse microbes differentially within parts of florets.

To address these questions, we first examined the abundance of microbes carried by two species of wild-caught foraging butterflies. We expected that the proboscis and tarsi carry more microbes than the thorax, based on their roles in foraging behaviour. We predicted that the larger butterfly species would carry more microbes, both because of greater body area and a presumed greater intake of nectar. Likewise, age and sex should be significant factors affecting the abundance of microbes carried by butterflies, because nectar intake varies with age (e.g., O'Brien et al., 2004) and sex (Boggs unpub.), and microbes are acquired from the environment over time. Second, we tested whether microbe abundance within florets affects the rate of dispersal among florets. Finally, we used a known bacterium to test the hypothesis that butterflies disperse microbes differentially among floral organs. We predicted that the floral nectaries have the greatest microbial diversity and abundance, followed by stigmas and anthers, based on expected differential contact by butterflies during nectar foraging.

METHODS

Study organisms and sites

We used *Glaucopsyche lygdamus* Doubleday, 1841 (Lepidoptera: Lycaenidae) and *Speyeria mormonia* Boisduval 1869 (Lepidoptera: Nymphalidae) as our pollinators because they differ in body size (forewing length: *G. lygdamus* 1.1–1.6 cm (Doubleday, 1841), *S. mormonia* 2.2–3.0 cm (Boggs, 1986). We focused on *Pyrrocoma crocea* (A. Grey) Green (Asteraceae) as the plant, because both butterfly species regularly visit it and other asters while foraging for nectar (Boggs, 1988). The florets are also large enough to be readily manipulated. The field site was located near the Rocky Mountain Biological Laboratory, Gothic, Gunnison County, Colorado (38°57′00″ N 106°59′11″ W, 2972 m ASL).

Distribution in wild-caught butterflies

We collected 10 *G. lygdamus* and 31 *S. mormonia* adults from the field site. We placed each individual in an autoclaved glassine envelope for transport to the lab to prevent contamination. We recorded species, sex and wing wear as a proxy for age. Wing wear was quantified using a 1–5 scale with 0.5 gradations, with 1.0 being fresh with incompletely dried wings and 5.0 being worn with extreme scale and wing edge damage (Boggs, 1987).

We used YM agar (yeast-malt extract agar: Difco YM broth, 2% plain agar) to isolate and culture yeasts and bacteria from the butterflies. Using sterile technique, we plated three samples from each collected individual by (1) walking the butterfly across the plate, (2) plating a wash of the thorax, and (3) extending the proboscis to touch the plate. To ensure that the butterflies' legs did not touch the plate while extending the proboscis with a sterilised pin, we held the butterflies so that their body was perpendicular to the agar surface while dragging the length of the proboscis across the plate. We counted the microbial colonies that grew after 2 days of incubation at room temperature. To ensure that colonies did not get recounted, we marked each colony with a permanent marker on the bottom of the plate while counting. We recorded a description of each colony and took photographs of colonies of a given description for reference. After the colonies were counted, we stored the plates in a refrigerator at 4°C to prevent plate overgrowth.

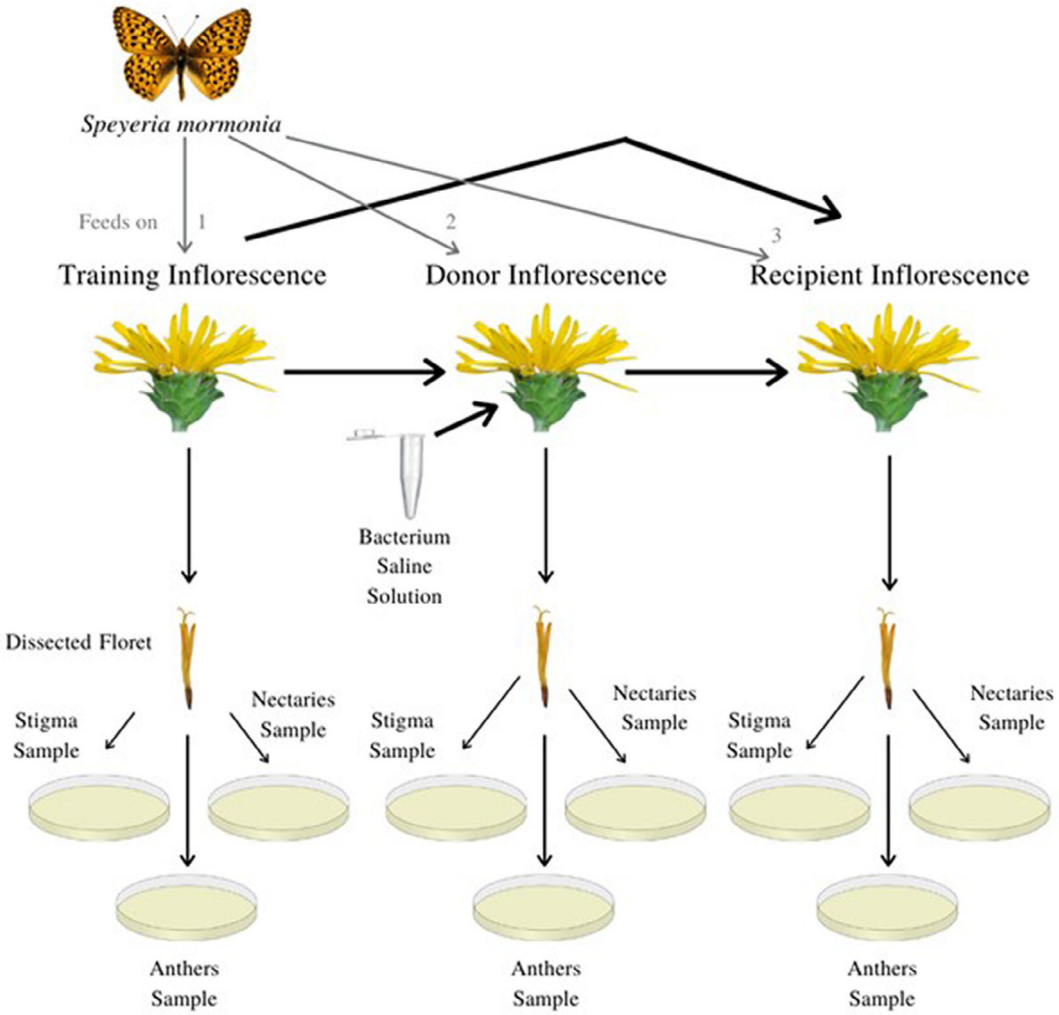

FIGURE 1 Illustrated depiction of methods.

Test bacterium isolation, culture and identification

We isolated a yellow bacterium collected from the tarsi and proboscis of wild-caught *S. mormonia* by 3X serial transfers on Petri plates with HiMedia nutrient agar containing: 3 g Amresco yeast extract, 10 g glucose, 20 g Amresco bacteriological agar, 5 g Bio-World bacto peptone, and 3 g Amresco malt extract, per litre of distilled water.

We extracted genomic DNA from our isolated culture using the DNeasy Blood and Tissue Kit (Qiagen, USA) using the manufacturer's protocol. Sanger sequencing of the 16S rDNA provided a preliminary identification of the bacterium as *Rhodococcus* sp. For a more precise identification, we used whole genome sequencing. Whole genome sequencing was carried out by Novogene on a Hi-Seq platform. Demultiplexed raw Illumina reads, and adapter sequences were trimmed using fastp (Chen et al., 2018) and a draft scaffold level genome was assembled using SPAdes (Bankevich et al., 2012) with default parameters. We then used NCBI Blast+ (Camacho et al., 2009) to identify the bacterium. We used blastn to search our genome sequence against the prokaryotic rRNA database and the nucleotide database to identify similar hits.

Butterfly microbe dispersal among florets and to floral organs

The trial arena consisted of a 3 × 6 m room with a large south-facing window. *Pyrrocoma crocea* inflorescences were placed near the window in a large floral pick with water to prevent wilting. We caught 26 *S. mormonia* adults and kept them in autoclaved glassine envelopes to starve overnight. We recorded sex and wing wear of each individual. To control for the possibility that the test bacterium was present on the butterfly or within the florets prior to the experiment, we first allowed the butterfly to feed on a training inflorescence in the arena (Figure 1). Immediately after feeding, we removed the floret on which the butterfly fed using sterile needle-tip forceps. We plated samples from the stigma, anthers and nectaries of that floret. To plate samples from the nectaries, we used sterile needle-tip forceps to remove and slice open the corolla tube to expose the nectaries.

For the experiments, we inoculated the donor inflorescence with the isolated test bacterium (Figure 1). We dispensed about 1 μL of a solution of 8.5% NaCl and bacterial cells using a micropipette into each floret. We allowed the inflorescence to dry. We then placed the donor inflorescence in the trial arena and allowed the butterfly to feed. Immediately after

feeding, we plated samples from the stigmas, anthers and nectaries of each donor floret that the butterfly fed on to get an initial microbial concentration. The donor inflorescence was then replaced with a recipient inflorescence, formerly the training inflorescence, and the butterfly was allowed to feed on the recipient inflorescence. Again, we removed and plated samples from the selected floral organs from the floret that the butterfly fed on. After incubating at room temperature for 1–4 days, we counted the number of colonies of the test bacterium.

Data analysis

All statistical methods were carried out using R v.3.6.1 (R Core Team, 2020).

Microbe abundance on butterfly parts

To determine if microbe abundance differed among butterfly parts, we used a generalised linear mixed model (GLMM) with a negative binomial distribution using the MASS package (Venables & Ripley, 2002). We used the number of colonies on butterfly body parts (thorax, feet, and proboscis) as the response variable, butterfly part, sex and wing wear (as proxy for age) as the fixed explanatory variables and the individual number as the random variable in our model.

Microbe dispersal among florets by butterflies

We used a generalised mixed model (GLMM) using a negative binomial distribution using the GLMM adaptive package (Rizopoulos, 2022) to account for overdispersion of our response variable. We used the number of microbial colonies on the recipient floret as the response variable, the number of microbial colonies on the donor floret and wing wear as the fixed explanatory variables, and the individual number as the random variable in our model. We selected the best model with the lowest AIC scores.

Butterfly microbe dispersal to floral organs

We used the aov function for a two-way ANOVA. We used the number of microbial colonies as the response variable and the floral organs (nectaries, anthers, stigma) and the floret type (donor versus recipient) as the explanatory variables. We used Tukey's HSD test for pairwise comparisons between means of our explanatory variables.

RESULTS

Microbial distribution in wild-caught butterflies

A total of 22 morpho-species of bacteria and fungi were plated from wild-caught butterflies. Butterfly tarsi and proboscides had significantly more microbes present than thoraces (Figure 2). The total overall microbial abundance was significantly greater for intermediate-aged and the oldest butterflies, compared to newly eclosed adults (Figure 2). *Glaucopsyche lygdamus* had significantly fewer microbes present than *S. mormonia* (Figure 2). Males also had fewer colonies than females (Figure 2).

Microbe dispersal among florets

The yellow bacterium used in the dispersal experiments was identified as *Rhodococcus fascians* (Tilford 1936) Goodfellow 1984 (NCBI accession number: near Y11196) (Table 1).

No *R. fascians* was detected on plates from floral organs of florets used as training flowers, indicating that *R. fascians* was not initially present either on the butterflies or the flowers used in the dispersal experiments. The sum of *R. fascians* abundance on the recipient floret's stigma, anthers and nectaries increased directly with increasing donor floret *R. fascians* abundance. (Figure 3). The youngest butterflies (wing wear class 2.5) transferred significantly fewer *R. fascians* to recipient florets than did older butterflies ($Z = -1.99$, $p = 0.046$).

Microbe dispersal to floral organs

The quantity of *R. fascians* was lower in recipient florets compared to the donor florets, indicating a reduction of microbial colonies between transfers (Figure 4). The quantity of *R. fascians* also differed among floral parts. Our Tukey HSD pairwise comparisons indicated that the number of microbial colonies between (a) nectaries and anthers and (b) stigma and nectaries were significantly different (Figure 4). The interaction between floret type (donor vs. recipient) and floral organ was not significant (full 2-way ANOVA with interaction term: floret type*floral organ, $F_{2,132} = 0.62$, $p = 0.54$), indicating no filtering of bacterial density among floret parts due to transfer by butterflies.

DISCUSSION

Microbial distribution in wild-caught butterflies

Total microbial abundance on wild-caught butterflies depended on body part, age, sex and species. Microbial abundance was greater on the tarsi and proboscis than on the thorax, supporting our hypothesis that abundance should be higher on body parts that are more likely to come into contact with flowers during foraging. Whether microbial abundance is correlated with abundance of pollen on butterfly body parts is still an open question, as few studies have examined pollen distribution. One, examining pollen distribution on *Euphydryas editha bayensis*, focused primarily on the head, thorax, abdomen and wings, ignoring the proboscis and tarsi (Murphy, 1984). In this butterfly, pollen from platform flower species was distributed across the butterfly's entire ventral body

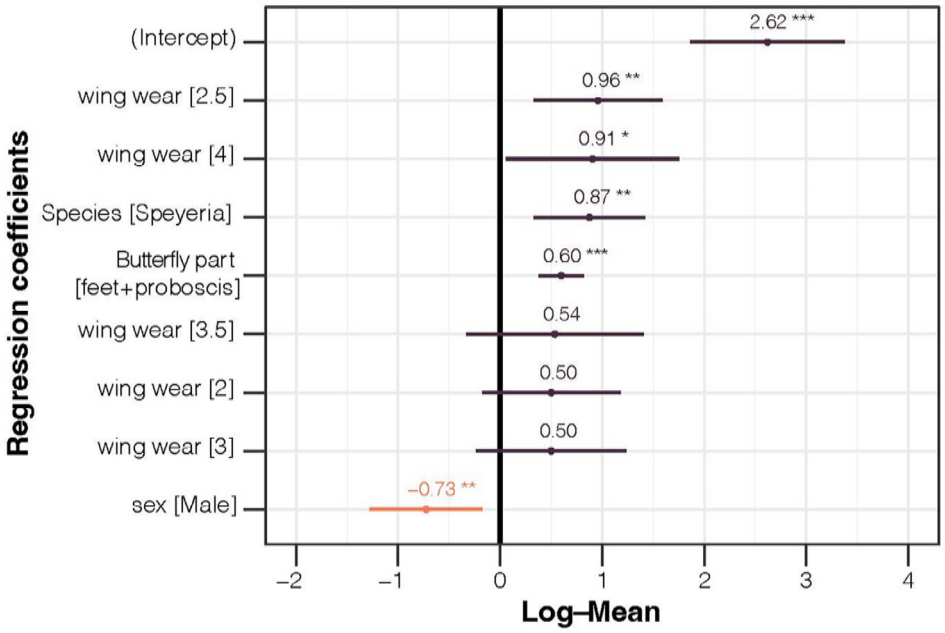

FIGURE 2 Regression coefficients for factors affecting the abundance of microbes carried by a butterfly. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. GLMM with individual butterfly as a random effect, and 71 degrees of freedom: intercept: $Z = 6.77$, $p \ll 0.001$; body part: $Z = 5.29$, $p \ll 0.001$; sex: −0.72, $p < 0.01$; species: $Z = 3.13$, $p < 0.002$; wing wear rating 2: $Z = 1.45$, $p = 0.15$ (ns); wing wear rating 2.5: $Z = 2.98$, $p < 0.003$; wing wear rating 3: $Z = 1.33$, $p = 0.18$ (ns); wing wear rating 3.5: $Z = 0.23$, $p = 0.23$ (ns); wing wear rating 4: $Z = 2.09$, $p < 0.04$.

TABLE 1 Results of BLAST of the isolated bacterium RNA scaffolds against Actinomycete RNA.

Scaffold	Match	% identity	Evalue
NODE_143_length_811_cov_1.303815	**Y11196.1**	96.104	9.17e−30
NODE_662_length_594_cov_1.411992	NZ_CP017014.1	95.623	0.0
	NZ_CP046257.1	94.482	0.0
	Y11196.1	94.463	0.0
NODE_1586_length_493_cov_2.105769	**Y11196.1**	98.936	0.0
	NZ_CP017014.1	98.377	0.0
NODE_5077_length_340_cov_1.638783	NZ_CP017014.1	99.412	5.03e−178
	NZ_LT906450.1	99.408	6.50e−177
	NZ_CP040719.1	99.408	6.50e−177
	NZ_LR134352.1	98.817	1.41e−173
	NZ_CP018082.1	98.817	1.41e−173
	NZ_CP015219.1	98.824	1.41e−173
	NZ_CP023714.1	98.521	6.55e−172
	NZ_CP019066.1	98.529	6.55e−172
	Y11196.1	98.235	33.05e−170

Note: **Y11196.1 is *Rhodococcus fascians***; NZ_CP017014.1 is *Rhodococcus sp*. WMMA185; NZ_CP046257.1 is *Gordonia sp*. 135; NZ_LT906450.1 is *Rhodococcus rhodochrous* strain NCTC10210; NZ_CP040719.1 is *Rhodococcus pyridinivorans*; NZ_LR134352.1 is *Nocardia asteroides* strain NCTC11293; NZ_CP018082.1 is *Nocardia mangyaensis* strain Y48; NZ_CP015219.1 is *Rhodococcus sp*. PBTS 1; NZ_CP023714.1 is *Rhodococcus ruber* and NZ_CP019066.1 is *Tsukamurella tyrosinosolvens* strain MH1.

surface. Nonetheless, our data show that butterflies do transport microbes on various body parts.

Female butterflies, as well as those that were in the youngest and oldest age categories, carried more microbes than did male or mid-aged butterflies in our study. This result supports our predictions that were based on a combination of differences in nectar intake in the lab and opportunity for older individuals to have accumulated more microbes. Similarly, *S. mormonia* had more microbes than *G. lygdamus*, again supporting our prediction based on differences in body size. This latter result is consistent with studies on Hymenoptera, showing

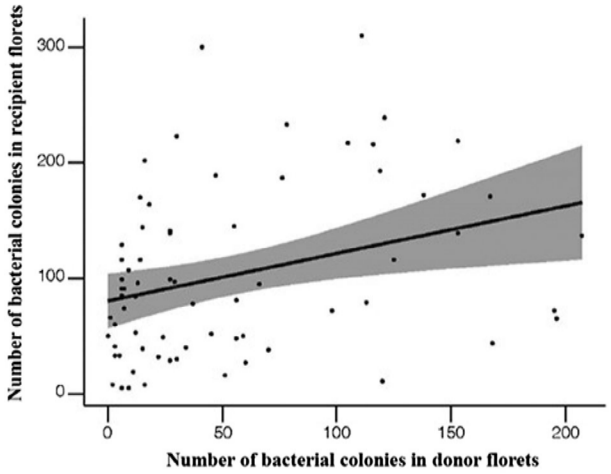

FIGURE 3 Transfer of *Rhodoccus fascians* to recipient florets of *Pyrrocoma crocea* by *Speyeria mormonia* was an increasing function of the abundance of *R. fascians* in donor florets. GLMM with individual butterfly as a fixed variable, and abundance in the donor floret and wing wear (an estimate of age) as fixed effects: donor abundance $Z = 5.36$, 63 df, $p < 0.0001$.

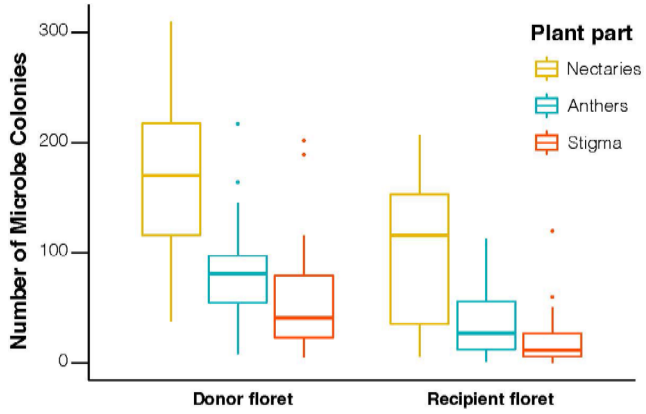

FIGURE 4 Mean number of colonies (±standard error) of *Rhodoccus fascians* from different floral organs including the nectaries, anthers and stigma. In a two-way ANOVA, donor florets contained more *R. fascians* than recipient florets ($F_{1,134} = 29.1$, $p \ll 0.001$), and floral organs differed significantly ($F_{2,134} = 37.3$, $p \ll 0.001$). More *R. fascians* were found on the nectaries than on the anthers (Tukey's HSD test: difference: 69.3 $p \ll 0.001$), or on the stigma (difference: 91.4, $p \ll 0.001$). The stigma and anthers did not differ significantly in presence of *R. fascians* (difference: −22.1, $p = 0.12$).

that larger bee species carry a greater abundance of microbes based on DNA analyses of body washes (Ushio et al., 2015).

Microbe dispersal among florets

Our results also provide direct evidence that butterflies can contribute to patterns of microbial abundance among and within flowers through dispersal of those microbes during nectar foraging. Transmission among flowers in our experiment depended on floret microbial abundance and butterfly age. Younger butterflies transmitted fewer *R. fascians* than did older butterflies. This result was unexpected, since butterflies were removed as soon as they started feeding. It is possible that older butterflies have more crevices in their proboscis and tarsi, due to wear and erosion, and hence more places for bacteria to lodge.

Microbe dispersal to floral organs

Nectaries contained significantly more microbes than the stigma or anthers in our donor and recipient florets. Contrary with our expectation, we found no evidence that butterflies filtered microbes differentially among floral organs, given that the interaction between floret type (donor vs. recipient) and floral organ was not significant. Our survey was done immediately after the butterfly visited the recipient floret, so the distribution pattern reflects deposition by the butterfly and not differential microbial reproduction. Microbial diversity differences among floral organs have been suggested previously to be due to environmental variation (Herrera et al., 2010; Pozo et al., 2012). Given that our butterflies dispersed microbes to the nectaries, anthers and stigmas, our results are consistent with studies that show that bee foraging affects microbial communities among different floral parts (Russell et al., 2019). Assuming that our results are representative of butterfly–flower–microbe interactions, butterflies could thus contribute to the maintenance of a core microbiome among floral organs (Albright & Martiny, 2018).

Caveats and broader implications

Our study was limited to two butterfly species and one plant species, plus for the transmission experiments, one bacterial species. Aside from the bright yellow colour of the colonies, it is noteworthy that *R. fascians* produces mucilage (Dhaouadi et al., 2020) that likely aids adherence of bacteria to the butterfly surfaces. Surveys of lepidopteran effectiveness in dispersal of a broader range of bacteria and fungi are therefore needed to verify the generality of our results. Nonetheless, this demonstration of effective transport of a microbial species, plus a community of microbial species resident on lepidopteran appendages likely to come into contact with flower parts, indicates that Lepidoptera are highly likely important participants in structuring the microbial communities of flower species that they visit.

In addition, we surveyed microbial abundance on butterfly body parts by streaking the parts (or wash of the thorax) on plates and counting colonies, rather than using molecular techniques. This will result in missing any bacteria or fungi that will not grow on plates, but the relative abundance of those microbes detected by streaking gives us an initial view of the microbial abundance relative to each body part. Since we were interested in relative abundance, our method should be sufficient. Further, identifying microbial communities on butterfly parts would likely have given us a very narrow view of what

Lepidoptera (or even our particular species) carry in other places and times.

Intriguingly, our study indicates that Lepidoptera may transport deleterious microbes when nectaring at flowers. Some strains of *R. fascians* are known to be plant pathogens (Dhaouadi et al., 2020), although we do not know the biology of our strain. Given that some butterflies are generalist pollinators (e.g., Scott, 1992; Zografou et al., 2020), the possibility of transmission exists for both pathogenic and mutualistic microbial species among plant species in the wild.

If our results regarding differences in microbial load as a function of pollinator size and age hold up across other lepidopteran species, then floral microbial community structure may depend not only on plant–pollinator network structure across time and priority effects of microbial colonisation (e.g., Jacquemyn et al., 2021), but also on pollinator body size and age. Likewise, dispersal of microbes by butterflies across a plant's flowering season could alter pollination networks and hence plant reproduction through changes in nectar qualities that affect pollinator preference. Since microbes change nectar composition and quality, pollinators may avoid certain flowers due to microbe colony composition (Good et al., 2014; Herrera et al., 2008; reviewed in Adler et al., 2021). Even if butterflies acquire few microbes during each flower visit, butterflies visit many flowers during foraging bouts and flowers receive many visits over the season (Williams & Thomson, 1998). Resulting changes in pollination networks may appear stochastic across space and time, with predictability based on nectar quality determined only by the rules of community assembly.

In sum, our study expands the insect pollination systems known to result in dispersal of microbes among flowers and pollinators, to include Lepidoptera. These species can be generalist pollinators, adding to the complexity of factors affecting microbial communities in flowers across both space and time. Future studies of the dynamics of such communities in the context of plant–pollinator networks will be crucial to a full understanding of dynamics of beneficial and pathogenic microbes and their effects on both plant and pollinator fitness.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

Data are available from the Dryad Digital Repository (doi:10.5061/dryad.0vt4b8h3n). Bacterial cultures have been deposited at the USDA Agricultural Research Service Culture Collection (NRRL – Northern Regional Research Laboratory), Peoria, Illinois (accession # B-65658). All DNA sequences have been deposited in NCBI Genbank (accession # PRJNA864660).

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