Host-specific spatial and temporal variation in culturable gut bacterial communities of dragonflies

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Many microbial communities are associated with specific animal hosts, with major impacts on the ecological and evolutionary dynamics of both partners. We analysed gut microbial communities of eight dragonfly species in southern India. Adult dragonflies are generalist opportunistic predators; thus, we expected to find relatively high individual variation but low host-specific variation in their gut community composition. However, we find that each host species has a distinct gut bacterial community, with sampling location and month playing a small but significant role in shaping community structure. These patterns suggest that dragonflies either specialize on subsets of available prey, or their guts impose differential selective pressures resulting in distinct communities.

Keywords: Community richness and dynamics, dragonfly, gut microbial communities, host–microbe interaction.

MICROBIAL communities associated with eukaryotic hosts can drive major ecological and evolutionary changes in host populations[1], playing important roles such as provisioning nutrients, preventing gut colonization by pathogens and aiding in immune function (for a recent review, see Engel and Moran[2]). To understand the ecology and evolution of these gut-associated communities, it is important to consider the processes that drive community assembly and maintenance. As with macrobial communities, we expect that microbial community assembly is determined by a combination of historical contingency (e.g. dispersal and speciation), stochastic processes (e.g. genetic drift) as well as selection (mediated via biotic and abiotic filters). However, dispersal and evolutionary change may play a much greater role in microbial communities due to high rates of passive dispersal and rapid rates of evolution[3]. For instance, if local community assembly is largely governed by stochastic processes (e.g. the order in which community members happen to colonize a specific gut), we would expect high variation in community structure between individual hosts as well as across host species. In contrast, if assembly is influenced largely by host-imposed selection, we would typically expect low variation between hosts of the same species, but higher variation between host species. Of course, both processes likely play a role in the assembly of most communities so that clearly determining the relative importance of both processes is difficult. Spatial and temporal variation in host populations or their gut physiology may further confound patterns of community assembly and maintenance. However, such variation remains relatively unexplored for microbial communities in general[4]. Thus, host gut-associated microbial communities are likely dynamic systems whose constancy, structure and function depend on microbial dispersal between hosts and the external environment, as well as interactions with the host and with other community members. Although the abundance and distribution of gut microbial communities have been explored in diverse insects, the impact of host

Figure 1. Map showing the location of the four sites from southern India.

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Table 1. Dragonfly hosts sampled

Dragonfly species	Sampling site	Sampling month	Sample size
Pantala flavescens	NCBS	January	10
	VIT	April	10
	Agumbe	October	5
	Agumbe	November	10
	Agumbe	January	10
Orthetrum sabina	NCBS	March	10
	VIT	April	10
	Agumbe	November	10
	Agumbe	January	8
Orthetrum pruinosum	Agumbe	November	7
	Agumbe	October	5
Potamarcha congener	NCBS	January	10
Neurothemis fulvia	Agumbe	January	8
Rhodothemis rufa	VIT	April	10
Trithemis pallidinervis	Pandalam	April	10
Urothemis signata	Pandalam	April	10

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population structure on the composition and diversity of the intestinal microflora remains poorly understood.

To begin to address these gaps, we analysed culturable gut bacterial communities of eight dragonfly species collected from four different sites in southern India (Figure 1 and Table 1). To determine short-term temporal changes in the community, we repeatedly sampled from one of these sites in different months. Dragonflies are important predators in both freshwater and terrestrial invertebrate food-webs and are believed to be generalist predators that feed on a wide diversity of insects[5]. Thus, their guts may serve as a reservoir of diverse bacteria. Dragonflies also tend to be strong fliers, allowing them to sample prey and associated microbes from a broad geographical area. Long-range dispersal of both host and symbionts as well as variation in prey communities set up the possibility that spatial and temporal variation in prey abundance may modulate the function and composition of gut microflora. However, there is no previous analysis of microbial associates of dragonflies. We found that the dragonfly gut contains a complex microbial community that is largely host species-specific, with relatively smaller but significant impacts of sampling site and month. The large impact of host species on the gut bacterial communities suggests that these are stably associated with each host.

Methods

Dragonfly collection and dissection

We collected dragonflies from four different sites (NCBS, VIT, Agumbe and Pandalam), using butterfly nets to sample 5–10 individuals per species per site per month (Figure 1; Table 1 and Supplementary Material (see online)). Each site had a small water body with either forest or planted urban vegetation nearby. Two sites (NCBS and VIT) are located in a research institute/university campus, and the other two are located near flooded paddy fields. We identified each specimen using various morphological characteristics[6–8]. For all host individuals, except those from Agumbe, we also noted host sex.

After collection, we gently surface-sterilized dragonflies with a cotton swab soaked in 70% ethanol and transferred them to a nylon mesh cage (24″ × 15″ × 15″) wiped with 70% ethanol. Within 4 h of capture, we euthanized each dragonfly by refrigerating it at 4°C when possible, or by keeping it on ice in a plastic bag for 15 min. For all collections, except from Agumbe, we measured body size proxies using Vernier calipers: head width; length and width of thorax, abdomen, hindwings and forewings, and body weight. After a second round of surface sterlization with 70% ethanol, we dissected the gut from mouth to rectum using sterilized dissection tools and preserved the rest of the body in 100% ethanol at 4°C for genetic analysis. We surface-sterilized the gut with a quick wash (1–3 sec) of 70% ethanol, followed by a wash with distilled water. The gut was further dissected into three regions: foregut, midgut and hindgut, and each region was cut into two halves (see Supplementary Material online). We kept both halves of each gut region in a 1.5 ml centrifuge tube containing 300 µl of 0.85% saline, stored at 4°C until culturing.

Culturing and identifying gut bacteria

For NCBS samples, we processed the gut contents within 10 h of dissection in a UV-sterilized Laminar hood. Other sampling sites did not have suitable laboratory facilities, and hence we transported these samples to NCBS on ice and plated them within 24 h of dissection. We suspended one half of each gut region (above) in 1 ml 0.85% saline and homogenized it using a sterile pestle. We diluted this

sample 1 : 100 in saline and spread-plated 100 µl aliquots on three different bacterial growth media poured in 60 mm × 15 mm petri plates : Luria–Bertani (LB) agar, MacConkey agar (HiMedia) and a chocolate base agar, each autoclaved at 121°C, 15 lb for 15 min. LB is a nutritionally rich medium that supports the growth of many common bacteria. MacConkey agar allows the growth of only Gram-negative bacteria and turns pink if the bacteria ferment lactose. Chocolate agar (beef extract, peptone, NaCl, 5% haemoglobin powder; HiMedia) is a rich medium that supports the growth of fastidious organisms and allows detection of haemolysin production. We preserved the remaining homogenized gut sample at –80°C after adding 1 ml 40% glycerol. For *Pantala flavescens* individuals collected from Agumbe in January, we also plated out a parallel set of plates (all three media) incubated in anaerobic conditions for 72 h (HiMedia Anaerobic Systems Mark II, 3.5 L).

For the other half of each gut region, we made a longitudinal incision with a fine wire loop to expose the inside of each tube-like region. We swabbed the exposed internal lumen and streaked the swab out on each of the three agar media mentioned above. We incubated all agar plates for 48–72 h at 30°C. After incubation, we identified distinct morphotypes using colony shape and size, pigmentation, viscosity, haemolysis on chocolate agar, growth rate and lactose fermentation. We streaked out morphologically distinct colonies from each medium on LB agar to select those that showed medium-specific morphology. Finally, we took each distinct morphotype and streaked it once more on the same medium from which it was isolated, to confirm that the morphology was stable. We allowed the colony to grow for 24–48 h at 30°C, and preserved the pure culture at –80°C in glycerol. To confirm that there was no external bacterial contamination during dissection, we swabbed the outside of each dissected gut before cutting it, and plated a suspension of the swab on all media. We did not observe any growth on these control plates.

To confirm the identity of each isolated bacterial morphotype, we isolated genomic DNA from –80°C stocks using the Promega genomic DNA isolation kit. Note that while we isolated hundreds of bacteria from each dragonfly gut, we only sequenced morphologically distinct colonies from each dragonfly species. We PCR-amplified and sequenced a 424 bp section in the hypervariable V4–V5 regions of the 16S rRNA gene (see Supplementary Material online). These are two of the most commonly sequenced regions for such analysis, allowing our work to be compared to similar efforts for other insects. We identified sequences using the SILVA[9], RDP Seqmatch[10] and Greengenes[11] databases. We aligned sequences using ClustalW[12]; generated a similarity matrix and delineated operational taxonomic units (OTUs) using various sequence similarity thresholds. We found that the databases did not contain OTUs that matched our OTUs at the species level (98% sequence similarity). Hence we classified OTUs at the genus level, using 92% dissimilarity as our threshold.

Genetic analysis of dragonflies

We dissected flight muscles from ethanol-preserved dragonflies and extracted host DNA (Promega Wizard kit), modifying the protocol as follows. We incubated samples with nuclei lysis solution for 1 h at 65°C and crushed the tissue with a sterile pestle. The partially lysed tissue is easily homogenized in this step; it is faster than grinding tissue with liquid nitrogen. We PCR-amplified and sequenced five mitochondrial regions of each dragonfly (Table S1, see Supplementary Material online), aligned sequences with ClustalW[12] and generated a maximum-likelihood tree (all sites Tamura–Nei model) with 200 bootstrap runs in MEGA6 (ref. 13). The tree topology was identical for each gene, and hence we show the tree generated for the LSU region as a representative tree.

Results

Gut community richness

We found similar number and diversity of OTUs (corresponding in our case to distinct genera) in the midgut and hindgut regions, whereas the foregut had very few OTUs. Hence, we pooled data from all three gut sections of each dragonfly for further analysis. From parallel plates cultured in aerobic and anaerobic conditions (for a subset of Agumbe dragonflies), we did not find any bacteria that were unique to the anaerobic culture. From a total of 561 sequenced clones isolated from the guts of 143 dragonfly individuals, we identified 300 bacterial OTUs (genera) differentiated at 92% sequence similarity (Table S2, see Supplementary Material online). To test whether we had adequately sampled the culturable gut bacterial diversity of each host species, we plotted taxon accumulation curves for each host using rarefaction analysis (sampling individuals with replacement), using the 'specaccum' function in the R package 'vegan'[15]. Overall, the expected total number of OTUs per species varied from ~20 to ~60. The rarefaction curves were nearly saturated in most cases (Figure S1, see Supplementary Material online), indicating that we could sample much of the culturable bacterial diversity from each dragonfly host species (per sampling site per month, where applicable). The curves also show that for the two host species with multiple sampling sites and temporal sampling (*Orthetrum sabina* and *Pantala flavescens*), community richness varied substantially across sites and month (Figure S1 *a* and S1 *b*, see Supplementary Material online).

A large fraction of OTUs (~120, 40% of total) were rare and found in only 1–5 dragonfly hosts (Figure 2). The rarest 21 OTUs were found only in a single host individual and belonged to common insect gut bacterial

Table 2. Analysis of variance (ANOVA) for factors affecting gut bacterial community richness (total OTUs)[a]

Model term	Df	Sums of squares	Mean squares	*F*	*P*
Host species	**7**	**686.6**	**98.09**	**9.993**	**< 0.001**
Host sex	2	5.4	2.7	0.275	0.7599
Month	**4**	**397.6**	**99.39**	**10.125**	**< 0.001**
Host species × sex	6	80.8	13.47	1.373	0.23116
Host species × month	2	54.5	27.24	2.776	0.06636
Host sex × month	**2**	**100.6**	**50.31**	**5.126**	**0.00732**
Residuals	119	1168.1	9.82		

[a]Significant terms are highlighted in bold.

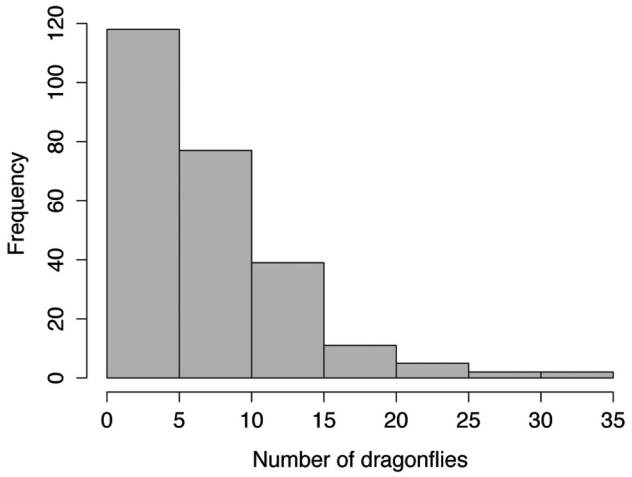

Figure 2. The frequency distribution of bacterial operational taxonomic units (OTUs) shown as a function of the number of host individuals in which they are found.

genera such as *Pantoea*, *Serratia*, *Streptomyces*, *Bacillus* and *Enterobacter*. Only 9 OTUs (3% of the total) were found in over 20 host individuals (~14% of sampled hosts) and this group included genera such as *Bordetella, Staphylococcus*, *Enterococcus*, *Bacillus*, *Gordonia*, *Klebsiella*, *Escherichia* and *Serratia*. Thus, most bacterial OTUs were found only in smaller subsets of sampled hosts and only a few were shared across many host individuals.

The total number of OTUs per dragonfly varied from 6 to 26. To identify factors contributing to this variation, we tested the impact of host species identity and sex, sampling month and sampling site on the total number of culturable bacterial OTUs (ANOVA, full model: total OTUs ~ host species * host sex * sampling site * sampling month). We found significant effects of host species, sampling month, and an interaction between host sex and month of sampling (Table 2). The largest difference in OTU richness was seen across host species, with some dragonfly species such as *P. congener* harbouring a median of 17 bacterial OTUs, and others such as *O. pruinosum* and *U. signata* harbouring ≤10 OTUs (Figure 3 *a*). For *P. flavescens* and *O. sabina*, we found that male and female gut community richness show different trends over time (Figure 3 *b*), indicating that host sex plays an important role in determining gut bacterial community richness. We also found that the number of total OTUs differed significantly over time, with samples collected in March showing maximum richness and those from October showing minimum richness (Figure 3 *c*). However, we found that sampling site did not have a significant impact on community richness (Figure 3 *d* and Table 2). This may be because host species identity and sampling month have a much stronger effect. Even when we restricted the dataset to *O. sabina* and *P. flavescens* (each of which was collected from three sites), we did not find a significant impact of sampling site on total OTUs. Instead, the best reduced model only contained significant effects of sampling month ($P < 0.001$) and month × host sex interaction ($P = 0.008$).

Why did host identity have such a large impact on gut community richness? We tested whether gut community richness was correlated with differences in host morphology. We found that the number of OTUs increased as a function of host body size, estimated by host body weight or thorax width (Figure 4; other proxies for body size gave similar results). However, the variation in body size was clearly distributed between rather than within host species: there was substantial variation in gut community richness within each host species despite little variation in body size (Figure 4). Thus, host-specific rather than individual differences in body mass were major contributors to the observed positive correlation between host body size and community richness.

Variation in gut bacterial community composition

Next we identified factors that impact the composition of gut bacterial communities across sampled dragonflies. We found that community composition (presence/absence of OTUs) was affected by host species, sampling site as well as sampling month. Hierarchical clustering showed that dragonfly individuals of the same species had the most similar gut community composition (Figure 5 *a*), and a permutation ANOVA showed that host identity explained 44% of the compositional variation (Table 3).

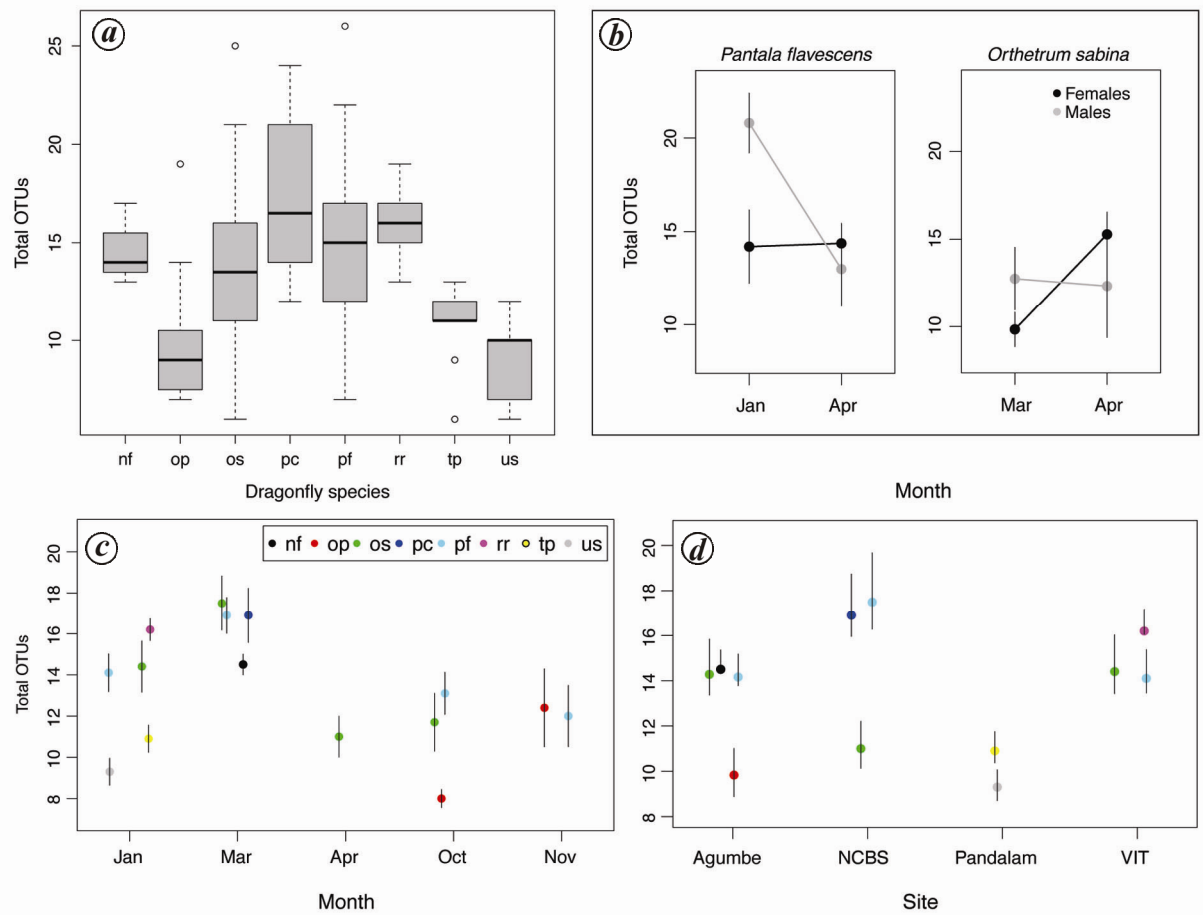

Figure 3. The total number of OTUs per gut as a function of (***a***) host species (box plots with quantiles), (***b***) interaction between host sex and sampling month, (***c***) sampling month and (***d***) sampling site. In ***b***–***d***, mean ± SE for each group is shown. In (***c*** and ***d***), points are coloured according to host species as indicated. Dragonfly host species names are abbreviated as follows: nf, *Neurothemis fulvia*; op, *Orthetrum pruinosum*; os, *Orthetrum sabina*; pc, *Potamarcha congener*; pf, *Pantala flavescens*; rr, *Rhodothemis rufa*; tp, *Trithemis pallidinervis*; us, *Urothemis signata*.

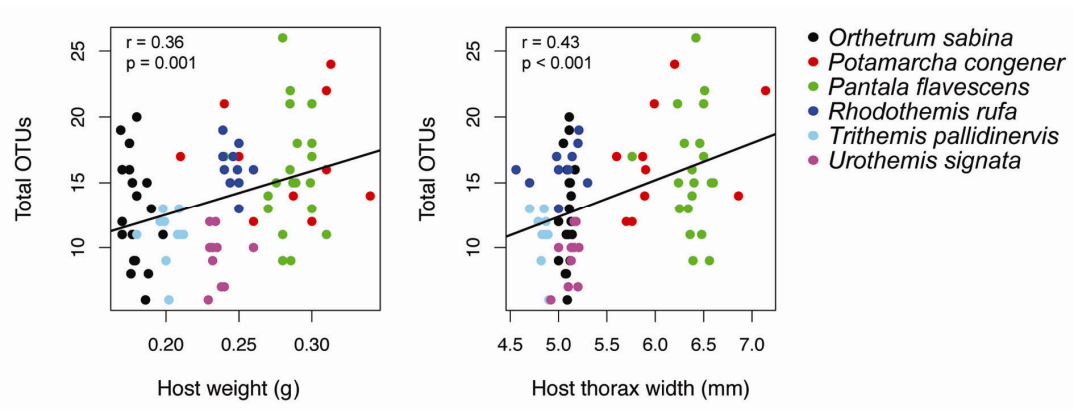

Figure 4. Correlation between total types of bacterial OTUs and two proxies of individual host size: host weight and maximum thorax width. Points are coloured according to the host species. Each panel shows the best-fit regression line, Pearson's correlation coefficient and associated *P* value. Morphological measurements were taken only for six of the eight host species analysed in this study.

For a given host species, individuals were further clustered according to the sampling site and sampling month (Figure 5 *a* and Table 3). Finally, interactions between host and sampling site, host and sampling month, as well as site and host sex each weakly affected community composition (explaining up to 5% of the variation; Table 3).

Table 3. Results of a permutation ANOVA for bacterial community membership[a]

Model term[b]	Df	Sums of squares	Mean squares	F	R^2	P
Host	**7**	**9.8284**	**1.40406**	**26.5275**	**0.4418**	**0.001**
Site	**2**	**1.5205**	**0.76024**	**14.3634**	**0.06835**	**0.001**
Month	**3**	**2.2781**	**0.75937**	**14.3471**	**0.1024**	**0.001**
Host sex	1	0.0161	0.01606	0.3034	0.00072	0.997
Host × site	**1**	**0.7235**	**0.72351**	**13.6696**	**0.03252**	**0.001**
Host × month	**2**	**1.1407**	**0.57037**	**10.7763**	**0.05128**	**0.001**
Host × sex	5	0.1985	0.03969	0.7499	0.00892	0.942
Site × sex	**1**	**0.2008**	**0.20083**	**3.7943**	**0.00903**	**0.001**
Month × sex	1	0.0413	0.04132	0.7806	0.00186	0.713
Residuals	119	6.2985	0.05293		0.28312	
Total	142	22.2464			1	

[a]The permutation ANOVA is a non-parametric analogue of a multiple analysis of variance (MANOVA), where data in a distance matrix are permuted across explanatory variables to test for their impact[30]. We calculated the distance matrix using Bray–Curtis dissmilarity and performed 1000 permutations of the data. [b]Significant model terms are highlighted in bold.

Thus, host species had the largest impact on gut bacterial community composition, with weaker but significant effects of sampling site and sampling month.

To test whether closely related dragonfly species have similar gut community composition, we generated a maximum likelihood phylogeny of our sampled dragonflies and compared the tree topology with that generated by gut community clustering. We found partial support for our hypothesis – *O. sabina* and *O. pruinosum* are evolutionarily closely related dragonflies that also had similar gut community composition (Figure 5 *b*). However, this correlation does not hold for other dragonflies. For instance, *N. fulvia* and *R. rufa* are closely related dragonflies with distinct gut communities. Thus, we do not find strong support for the hypothesis that closely related dragonfly species have similar gut bacterial communities.

Major orders of culturable gut bacteria

To test whether specific orders of bacteria were relatively over or under-represented across host species, we determined the taxonomic distribution of bacteria in gut communities, assigning each named OTU to a bacterial order (33 OTUs could not be identified from 16S rRNA databases). We found substantial spatial, temporal and host-associated dynamics in the occurrence of bacterial orders across host guts (Figure 6). It is important to note that these data do not indicate relative abundance in terms of the fraction of total bacterial cells. Instead, they reflect the relative diversity within each bacterial order and its contribution to the total diversity of the gut bacterial community.

Within each host species, there was no apparent sexual dimorphism in community composition, suggesting that the observed sexual differences in community richness were driven by an overall increase or decrease in bacterial diversity, rather than major changes in specific groups of bacteria. We also tested whether the number of bacterial OTUs assigned to major orders was correlated across individual hosts (e.g. whether increasing representation of Enterobacteriales in host guts was consistently associated with a change in representation of another order). All significant correlations between orders ($P < 0.001$) were positive ($0.25 < r < 0.7$), indicating that representation of members of many pairs of bacterial orders increased concurrently (Table 4). For instance, Actinomycetales and Aeromonadales showed a strong positive correlation ($r = 0.7$), as did Burkholdariales and Pseudomonadales ($r = 0.6$). These associations may result from facilitative interactions between bacteria from different orders, or indicate a common dietary (or other environmental) source.

Our data suggest that the composition of the gut bacterial community changes substantially across different populations of the same host species, potentially reflecting local variation in diet (Figure 6). For instance, for *P. flavescens* and *O. sabina* about 40% of community richness was attributed to Enterobacteriales, which is a commonly occurring bacterial order in many insect guts[15]. However, *P. flavescens* individuals from Agumbe – especially those collected in October and November – had much higher proportion of Enterobacteriales (50–80%), whereas *O. sabina* from VIT had only about 10–20% Enterobacteriales. Similarly, Burkholdariales was found in many *P. flavescens* individuals from Agumbe, but was rare or absent in *P. flavescens* sampled from the other two sites; and Flavobacteriales was frequently found in *O. sabina* collected from VIT, but not from NCBS or Agumbe.

Comparing across host species, we observed that Enterobacteriales, Xanthomonadales, Bacillales, Lactobacillales, Actinomycetales and Burkholderiales were generally well represented across hosts, although to varying degrees (Figure 6). These orders are all common in insect gut communities and include opportunistic insect

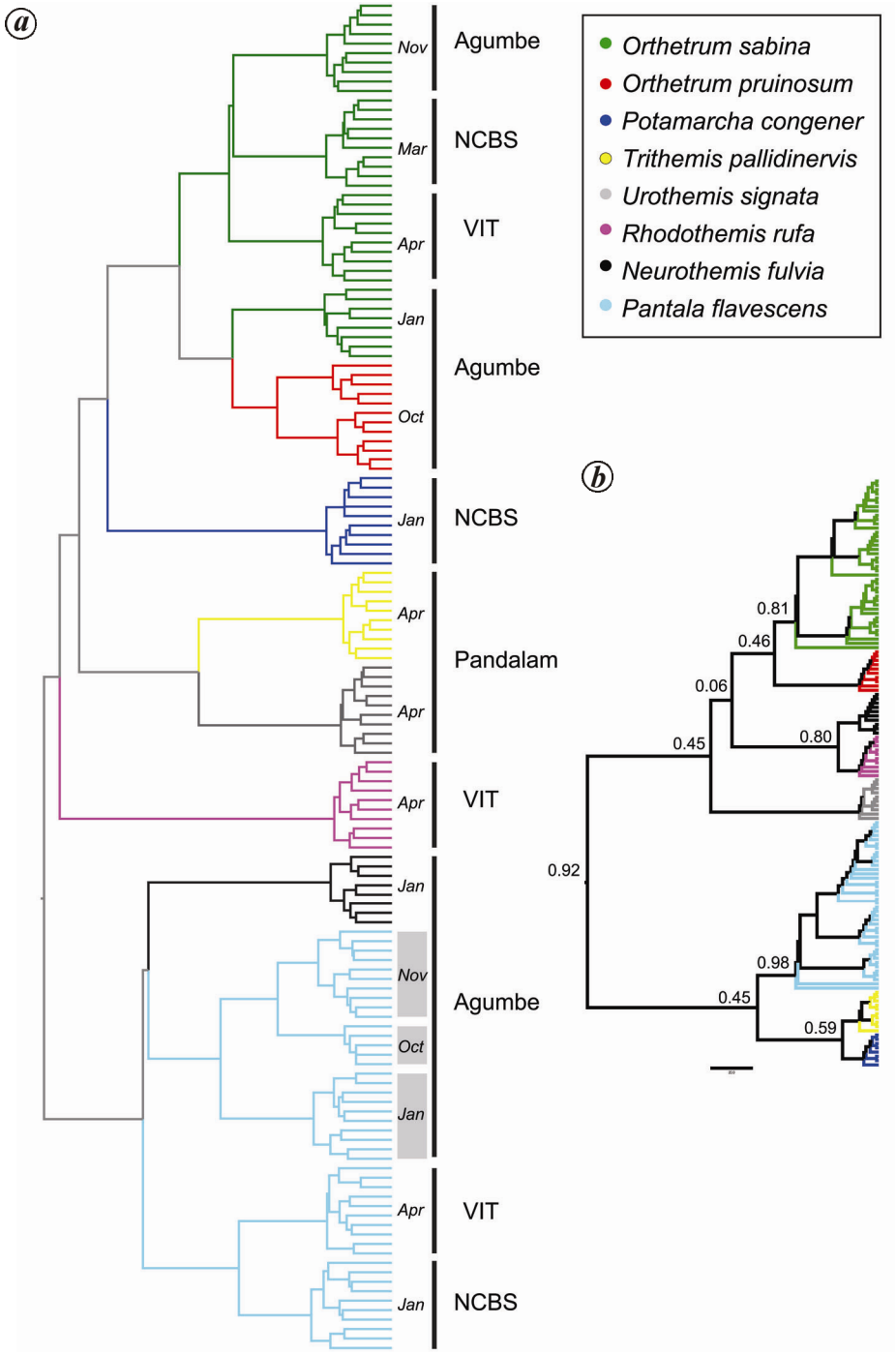

Figure 5. ***a***, Hierarchical clustering of gut community composition. Each branch in the cluster dendrogram represents an individual host. The hierarchical clustering is based on Ward's dissimilarity calculated using a presence/absence matrix for each OTU found in each host individual. Branches are coloured by host species, and sampling month and site are indicated for each cluster. ***b***, Maximum likelihood phylogeny of all sampled dragonfly individuals showing bootstrap support for all major nodes. Branches are coloured by species as indicated.

pathogens as well as mutualists; though in many cases their precise role is not known[15]. In contrast, Rhizobiales, Flavobacteriales and Aeromonadales were consistently present only in a few host species, and their roles in a carnivorous insect gut are unclear. Oceanospirillales was quite rare and observed only in a few individuals of *P. congener*, *T. pallidinervis* and *U. signata*. Finally, the rarest order was Neisseriales, observed only in three individuals of *O. sabina* collected from Agumbe in November. It is possible that the presence of these rare orders indicates a specific association with their hosts, but currently there is no evidence to support or reject this hypothesis.

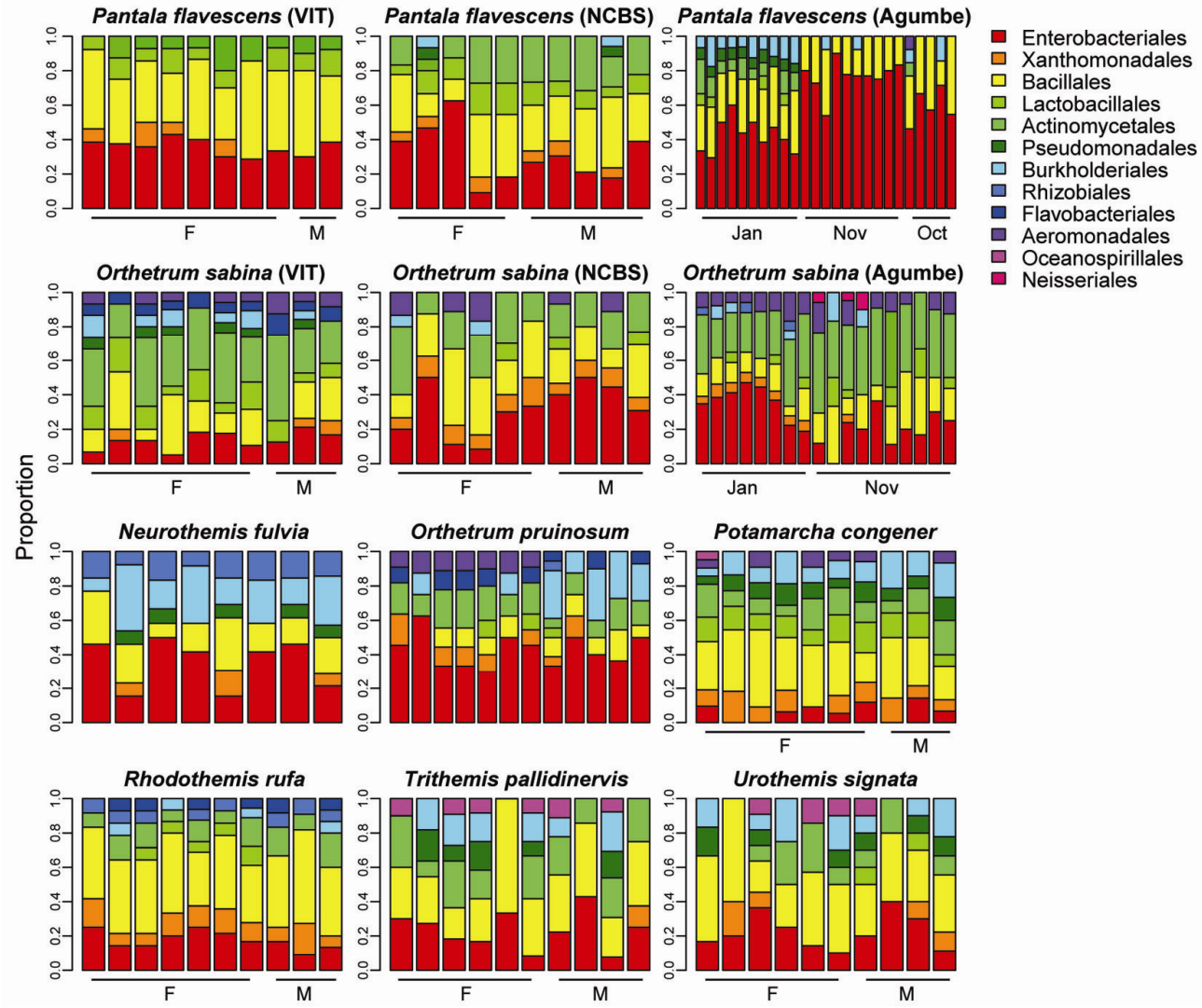

Figure 6. Distribution of orders of culturable gut microbes shown for each host species. Each bar represents an individual dragonfly, with sections coloured according to the proportion of bacterial OTUs belonging to each order. Where data are available, host individual sex is indicated (F = females, M = males). For *P. flavescens* and *O. sabina*, individuals are grouped based on the sampling site. For Agumbe samples, the sampling month is indicated below the bars.

Table 4. Correlation between represented orders across host individuals[a,b]

Order	Ent	Xan	Bac	Lac	Act	Pse	Bur	Rhi	Fla	Aer	Oce	Nei
Ent	1.00	–0.23	–0.10	–0.23	–0.16	–0.25	–0.14	0.04	–0.17	–0.06	–0.23	–0.05
Xan		1.00	**0.31**	**0.35**	0.08	0.10	0.03	0.15	0.10	0.11	–0.12	–0.05
Bac			1.00	**0.40**	0.01	0.19	0.03	0.03	–0.09	–0.25	–0.01	–0.08
Lac				1.00	**0.28**	0.22	0.00	–0.23	0.18	0.02	–0.10	–0.06
Act					1.00	–0.04	–0.16	–0.17	0.23	**0.70**	0.01	**0.34**
Pse						1.00	**0.61**	–0.01	–0.08	–0.10	**0.28**	–0.09
Bur							1.00	**0.29**	0.03	–0.16	0.13	–0.07
Rhi								1.00	0.06	–0.09	–0.11	–0.06
Fla									1.00	0.10	–0.13	–0.07
Aer										1.00	–0.12	**0.35**
Oce											1.00	–0.04
Nei												1.00

[a]Cell values show the correlation coefficient between the number of OTUs assigned to each pair of bacterial orders indicated. [b]Significant correlations ($P < 0.001$) are highlighted in bold.

Shared and unique bacterial genera in the dragonfly gut microbial community

To understand the role of sampling site and host species on unique and shared bacterial community members, we estimated the number of bacterial genera that were shared across hosts and sampling sites. We found that at both NCBS and VIT, about 15% of bacterial genera were shared across the three host species sampled at each location (Figure 7 *a*). In Agumbe, the proportion of shared genera was slightly lower (8%), whereas in Pandalam the proportion was as high as 41%. It is possible that the low proportion of shared genera in Agumbe samples reflects temporal variation in community composition, since we pooled data from all sampling months for this analysis. However, the cause of the extremely low fraction of unique genera for hosts from Pandalam remains unclear. Since we do not have host species common to Pandalam and the other sites, the effect of sampling site could be confounded by host identity. Future analysis of the gut flora of *T. pallidinervis* and *U. fulvia* from other sites would allow us to test whether these two host species generally tend to share most of their gut microbial communities.

For the two host species sampled from different sites, we found that ~20% of bacterial genera were shared across sites (Figure 7 *b*). Thus, about one-fifth of the gut community genera was similar regardless of spatial location, and may potentially reflect community members that are functionally associated with the host. However, presently we cannot reject the alternative hypothesis that these shared genera are associated with common prey species used by a host across different sites.

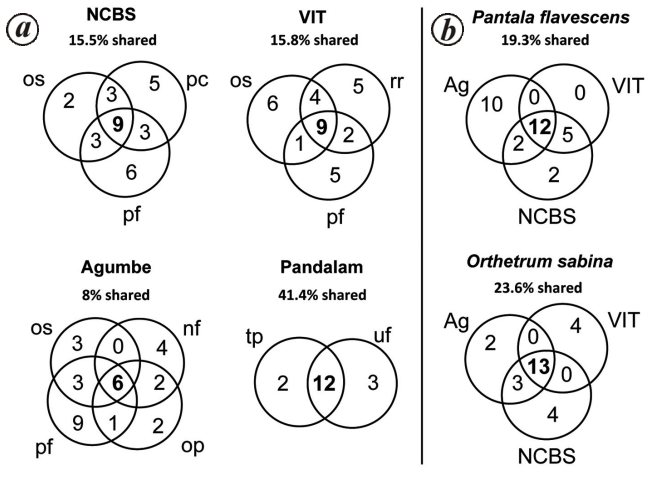

Figure 7. ***a***, Venn diagrams show bacterial genera shared by all dragonfly hosts from each sampling site. Hosts are abbreviated as in Figure 3. For Agumbe samples, only a subset of all possible comparisons is shown for clarity. ***b***, Venn diagrams show genera shared between dragonfly hosts from different sampling sites; Ag = Agumbe. In both panels, the proportion of total genera shared between all samples (% shared) is indicated below the sampling site or host species name.

Discussion

In the past few decades, a large number of studies have analysed insect gut microbial communities in an effort to determine the prevalence of different microbes, their potential roles, and their impact on host ecology and evolution. Host–microbe interactions can be affected by dispersal, selection and historical contingency from both the host and microbial perspective. Hence, to better understand how such interactions may be established and maintained, it is important to consider spatial and temporal variation in host and microbial populations. As a step in this direction, we analysed the impact of host species, sampling site and sampling time on culturable gut bacterial communities of dragonflies. We found that gut community richness and composition are both largely influenced by host species identity, even for different host species collected from the same location. Similar patterns of host-specific gut communities were observed for three sympatric species of tsetse fly from Uganda[16]. The parallel occurrence of species-specific gut communities is especially interesting because in contrast to dragonflies, tsetse flies are obligate specialists that feed on vertebrate blood, although they may show behavioural preference for different vertebrates[16].

Gut communities of host species collected from the same site could differ due to two non-exclusive reasons: (1) Hosts specialize on distinct prey species and gut communities reflect different microbes that are passively acquired with the prey (neutral community assembly). (2) Hosts consume similar prey, but gut communities diverge due to host-specific selection pressures in the gut environment (community assembly shaped by selection). Previous work suggests that some dragonflies specialize on butterflies[17,18], other dragonflies[19] or large-sized prey[20]. However, quantitative analyses of adult diet and potential resource partitioning are rare. A recent analysis of two Libellulid dragonflies showed substantial species-specific variation in foraging behaviour[21]. An analysis of microhabitat use and behaviour of three sympatric *Orthetrum* species also suggested niche partitioning among them[22]. It is plausible that such variation may result in sympatric host species sampling distinct subsets of available prey. For the dragonflies we sampled, we found that larger host species tend to harbour richer gut bacterial communities. This correlation may arise if larger dragonflies have larger guts and provide more distinct niches that different bacteria can occupy. Alternatively, if small dragonfly species are constrained by the size of the prey they can capture, larger dragonflies may access a larger subset of available prey species. The diverse prey base may in turn directly select for or neutrally lead to higher gut community richness. Different dragonfly species may also impose differential selection pressures on their gut communities, either through different aspects of gut physiology (e.g. pH) or via distinct immune systems. To

distinguish between these possibilities, we need information on the dietary habits of dragonflies, the microbes associated with their prey, and the nature of selection imposed by the physiological and spatial aspects of host guts. Unfortunately, such data are not available for our focal host species.

As with host-specific variation, temporal and spatial variation in dragonfly gut communities may also be attributed to neutral or selective processes, or a combination of the two. For instance, variable prey availability across different habitats or months could drive spatial and temporal variation in predator gut communities. Adult dragonfly diet is known to covary with prey abundance on short timescales[23], although dietary changes for our specific sampled species are unknown. Alternatively, gut microbial communities may be acquired through contact with conspecifics from the same population, although this is unlikely since dragonflies are not known to engage in extensive social interactions and parental care. Gut bacterial communities of humans show extensive temporal and geographical variation (see Parfrey and Knight[24] and other recent reviews), but similar data for insect species are rare.

Our work complements recent studies analysing the various factors that determine insect gut bacterial communities. A meta-analysis of 16S rRNA-based surveys of insect gut microbial communities showed that host diet significantly affects gut microbial communities, with a relatively low impact of host taxonomy[15]. Although host diet had the largest impact on community composition, the magnitude of the effect was not very high (22% variation explained). Analysis of ant gut microbial communities also showed that herbivorous ants have distinct communities compared to carnivorous ants, and that different predatory ants have similar gut microbiomes[25]. In contrast, two other large studies found that host species and taxonomy – rather than diet – are major determinants of gut bacterial composition[26,27]. Some of this variation across insects and studies may be attributed to additional ecological factors that were not considered. For instance, we found weak but significant impacts of sampling site and sampling month on dragonfly gut community richness and composition. In contrast, in the bollworm, host plant differences contributed significantly to the gut microbiome regardless of sampling site[28]. The gut microbiome also reflected the phyllosphere microbiome of each plant, suggesting that the host plant largely determines the gut microbial community of this generalist pest. Other confounding factors may include host sex: e.g. female bollworm microbiomes were less diverse than males[28]. We also found that community richness and composition of female and male dragonflies varied differentially as a function of sampling month and site respectively. Such sex-specific variation in gut community structure may arise if males and females consume distinct resources, or if they impose different selection pressures on the gut community.

Insect gut microbial communities tend to have low alpha diversity but high beta diversity. A previous analysis of 39 insect species found that a single phylotype dominates each community (~55% of total bacteria), but only 6% of the total phylotypes is shared between more than five insect hosts[26]. As a result, many bacterial phylotypes (69%) are unique to specific insect species. Our work supports this pattern, showing an abundance of rare OTUs and large differences in gut community composition between host species. Although we could not reliably estimate OTU abundance in our study, ~40% of the culturable bacterial OTUs were shared by very few (1–5) dragonfly hosts. For a given host, ~20% of bacterial genera were shared across sampling sites, and within a sampling site 8–41% genera were shared across host species. Further work to characterize the gut microbial communities using culture-independent methods is necessary to draw firm conclusions about the alpha and beta diversity of dragonfly gut bacterial communities. Also, we have probably underestimated taxonomic diversity in dragonfly gut bacterial communities, because we could only classify taxa to genus level. As demonstrated in a recent systematic analysis of reference 16S rRNA databases, complete taxonomic classification of bacteria (including higher level classification to phyla) requires longer sequences[29].

In summary, we have demonstrated that dragonflies harbour rich gut bacterial communities that are strongly influenced by host species identity, along with significant spatial, temporal and host sex-specific variation. Although our analysis was limited to culturable bacteria, consistent sampling and culturing methods allowed us to address our central questions about the factors shaping variation in gut bacterial communities of a major insect predator. Our data suggest that dragonfly guts serve as major reservoirs of a diverse array of bacterial species, including opportunistic insect and human pathogens. We hope that our work spurs further interest in understanding the establishment and persistence of gut microbial communities with respect to their divergence within and across closely related host species.

1. McFall-Ngai, M. *et al.*, Animals in a bacterial world, a new imperative for the life sciences. *Proc. Natl. Acad. Sci. USA*, 2013, **110**, 3229–3236.
2. Engel, P. and Moran, N. A., The gut microbiota of insects – diversity in structure and function. *FEMS Microbiol. Rev.*, 2013, **37**, 699–735.
3. Barberán, A., Casamayor, E. O. and Fierer, N., The microbial contribution to macroecology. *Front. Microbiol.*, 2014, **5**, 203.
4. Nemergut, D. R. *et al.*, Patterns and processes of microbial community assembly. *Microbiol. Mol. Biol. Rev.*, 2013, **77**, 342–356.
5. Corbet, P. S., *Dragonflies: Behavior and Ecology of Odonata*, Cornell University Press, USA, 1999.
6. Subramanian, K. A., *Dragonflies and Damselflies of Peninsular India – A Field Guide*, Indian Academy of Sciences, Bengaluru, 2005.
7. Abbott, J. C., Odonata Central: an online resource for the distribution and identification of sOdonata, 2006; www.odonatacentral.org.

8. Smallshire, D. and Swash, A., *Britain's Dragonflies: A Field Guide to the Damselflies and Dragonflies of Britain and Ireland*, Princeton University Press, USA, 2010.
9. Quast, C. *et al.*, The SILVA ribosomal RNA gene database project: improved data processing and web-based tools, 2013, *Nucleic Acids Res.*, **41**, D590–D596.
10. Cole, J. R. *et al.*, Ribosomal database project: data and tools for high throughput rRNA analysis. *Nucleic Acids Res.*, 2014, **42**, D633–D642.
11. DeSantis, T. Z. *et al.*, Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.*, 2006, **72**, 5069–5072.
12. Larkin, M. A. *et al.*, ClustalW and ClustalX version 2.0, *Bioinformatics*, 2007, **23**, 2947–2948.
13. Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S., MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.*, 2013, **30**, 2725–2729.
14. Colman, D. R., Toolson, E. C. and Takacs-Vesbach, C. D., Do diet and taxonomy influence insect gut bacterial communities? *Mol. Ecol.*, 2012, **21**, 5124–5137.
15. Oksanen, J. *et al.*, vegan: Community Ecology Package; 2015, http://CRAN.R-project.org/package=vegan.
16. Aksoy, E. *et al.*, Analysis of multiple tsetse fly populations in Uganda reveals limited diversity and species-specific gut microbiota. *Appl. Environ. Microbiol.*, 2014, **80**, 4301–4312.
17. Larsen, T. B., Butterflies as prey for *Orthetrum austenti* (Kirby) (Anisoptera: Libellulidae), *Not. Odonatol.*, 1981, **1**, 130–133.
18. Owen, D. F. *Orthetrum austini* Kirby, a specialist predator of butterflies (Anisoptera: Libellulidae), *Not. Odonatol.*, 1993, **4**, 34.
19. Dunkle, S. W., *Dragonflies of the Florida Peninsula, Bermuda and the Bahamas*, Scientific Publishers, 1989.
20. White, D. S. and Sexton, O. J., The Monarch butterfly (Lepidoptera, Danaidae) as prey for the dragonfly *Hagenius brevistylus* (Odonata, Gomphidae), *Entomol. News*, 1989, **100**, 129–132.
21. May, M. L. and Baird, J. M., A comparison of foraging behavior in two 'percher' dragonflies, *Pachydiplax longipennis* and *Erythemis simplicicollis* (Odonata: Libellulidae), *J. Insect. Behav.*, 2002, **15**, 765–778.
22. Khelifa, R. *et al.*, Niche partitioning in three sympatric congeneric species of dragonfly, *Orthetrum chrysostigma, O. coerulescens anceps*, and *O. nitidinerve*: the importance of microhabitat. *J. Insect. Sci.*, 2013, **13**, 1–17.
23. Baird, J. M. and May, M. L., Foraging behavior of *Pachydiplax longipennis* (Odonata: Libellulidae). *J. Insect. Behav.*, 1997, **10**, 655–678.
24. Parfrey, L. W. and Knight, R., Spatial and temporal variability of the human microbiota, *Clin. Microbiol. Inf.*, 2014, **18**, 5–7.
25. Anderson, K. E. *et al.*, Highly similar microbial communities are shared among related and trophically similar ant species. *Mol. Ecol.*, 2012, **21**, 2282–2296.
26. Jones, R. T., Sanchez, L. G. and Fierer, N., A cross-taxon analysis of insect-associated bacterial diversity. *PLoS ONE*, 2013, **8**, e61218.
27. Yun, J.-H. *et al.*, Insect gut bacterial diversity determined by environmental habitat, diet, developmental stage, and phylogeny of host. *Appl. Environ. Microbiol.*, 2014, **80**, 5254–5264.
28. Priya, N. G., Ojha, A., Kajla, M. K., Raj, A. and Rajagopal, R., Host plant induced variation in gut bacteria of *Helicoverpa armigera*. *PLoS ONE*, 2012, **7**, e30768.
29. Yarza, P. *et al.*, Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nature Rev. Microbiol.*, 2014, **12**, 635–645.
30. Anderson, M. and Braak, T. C., Permutation tests for multifactorial analysis of variance. *J. Stat. Comput. Simul.*, 2003, **73**, 85–113.

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