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Fruit odorants mediate co-specialization in a multispecies plant–animal mutualism

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Despite the widespread notion that animal-mediated seed dispersal led to the evolution of fruit traits that attract mutualistic frugivores, the dispersal syndrome hypothesis remains controversial, particularly for complex traits such as fruit scent. Here, we test this hypothesis in a community of mutualistic, ecologically important neotropical bats (*Carollia* spp.) and plants (*Piper* spp.) that communicate primarily via chemical signals. We found greater bat consumption is significantly associated with scent chemical diversity and presence of specific compounds, which fit multi-peak selective regime models in *Piper*. Through behavioural assays, we found *Carollia* prefer certain compounds, particularly 2-heptanol, which evolved as a unique feature of two *Piper* species highly consumed by these bats. Thus, we demonstrate that volatile compounds emitted by neotropical *Piper* fruits evolved in tandem with seed dispersal by scent-oriented *Carollia* bats. Specifically, fruit scent chemistry in some *Piper* species fits adaptive evolutionary scenarios consistent with a dispersal syndrome hypothesis. While other abiotic and biotic processes likely shaped the chemical composition of ripe fruit scent in *Piper*, our results provide some of the first evidence of the effect of bat frugivory on plant chemical diversity.

1. Background

Interactions between fruiting plants and frugivores generate and maintain phenotypic and species diversity [1]. While effective seed dispersal by animals is critical to fitness for thousands of angiosperm species [2], frugivores benefit from the nutrients and spatio-temporal predictability of fleshy fruits. These mutual advantages are thought to explain both the evolution of animal-mediated seed dispersal [1] and the staggering diversity of fruit traits that attract seed-dispersing frugivores [2,3]. To explain the apparent correspondence between fruit traits and frugivore phenotypes, the ‘dispersal syndrome’ hypothesis [4–6] posits fruit traits evolved adaptively to match the behaviour, physiology and morphology of mutualistic frugivores. Although this hypothesis has been invoked to explain fruit presentation, colour (e.g. [7–9]), size (e.g. [10,11]) and odour (e.g. [12,13]) differences among plant taxa, comparative tests yield mixed support, sometimes highlighting the roles of phylogenetic history and abiotic factors (e.g. [3,14,15]), or supporting the selective role of seed dispersers (e.g. [6]). Evolutionary dynamics within plant–frugivore mutualisms may also favour multispecies networks, in which sets of mutualistic species converge and specialize on a core set of traits instead of coevolving [16].

Most studies of the dispersal syndrome hypothesis have focused on trait complexes (e.g. fruit colour, size, shape) influenced by distantly related frugivores (e.g. birds versus mammals) [17–19]. Though informative about the effect of divergent frugivore behaviors and physiologies on fruit trait macroevolution, spatial and phylogenetic effects can dominate these patterns [3], obscuring how specific interactions, or interactions along a mutualistic continuum, influence fruit trait evolution. Here, we focus on a system of sympatric, mutualistic species: pepper plants (*Piper*) and short-tailed fruit bats (*Carollia*), which communicate primarily via one type of fruit cue (scent) and frugivore sensory modality (olfaction) [20], and constitute a multi-species gradient of ecological interactions. With approximately 2000 species across its pantropical distribution, *Piper* is megadiverse, abundant, economically and ecologically important [21]. In the Neotropics, most *Piper* produce small-seeded, greenish infructescences that become fragrant when ripe, purportedly to attract seed-dispersing bats [22]. Accordingly, the seeds of many neotropical *Piper* are primarily dispersed by short-tailed fruit bats (*Carollia*), which rely on *Piper* as a main food resource [23] and primarily use olfaction to find fruit [20,24–26].

Fruit scents are a mélange of volatile organic compounds (VOCs), secondary metabolites produced during ripening. Most studies of the macroevolutionary patterns of fruit scent have been qualitative, with few tests of the effect of scent differences on frugivore attraction (e.g. [27,28]). Therefore, little is known about how interspecific variation in VOCs mediates mutualistic interactions with frugivores. In *Piper*, chemical trait selection via mutualisms with bats is likely because these plants gain fitness benefits through greater ranges of seed dispersal [29], higher germination performance after defaecation by *Carollia* [30], and lower herbivore damage via higher *Piper* diversity due to bat dispersion [31]. But, with a variety of functions, including reducing bacterial and fungal attacks [32,33], VOCs could be byproducts of fruit maturation instead of signals adapted to target mutualistic frugivores [27]. We use the *Piper*–*Carollia* system to investigate the adaptive hypothesis that ecological interactions with *Carollia*, and bat behavioural preferences for specific chemical signals, shaped the evolution of fruit scent across *Piper*. Applying an integrative approach within an ecological community, we quantified the chemical composition of ripe fruit scent across 22 *Piper* species (Aim 1), characterized the diet of three *Carollia* species (Aim 2), determined the preference of *Carollia* for specific chemicals found in *Piper* ripe fruit scent (Aim 3), and quantitatively linked bat diet and chemical preferences to the adaptive evolution of *Piper* fruit scent chemistry (Aim 4).

2. Methods

This study was conducted at La Selva Biological Station, Costa Rica, where *Piper* is highly diverse (over 50 recognized species [34]), and three *Carollia* species (Chiroptera: Phyllostomidae; *C. castanea*, 11 g; *C. sowellii*, 18 g; and *C. perspicillata*, 21 g) are some of the most abundant bats year-round and coexist with approximately 62 other bat species. Research was conducted under Costa Rican permits SINAC-SE-GCUS-PI-R-117-2015, 038-2017-ACC-PI, ACC-PI-030-2018, R-041-2016-OT-CONAGEBIO. All procedures for bat capture and handling were approved by the University of Washington's and Stony Brook University's Institutional Animal Care and Use Committees (UW 4307-02; SBU 2013-2034-R1-8.15.19-BAT).

(a) Aim 1: quantify the chemical composition of ripe fruit scent

To quantify the VOCs that constitute their scent, we collected ripe *Piper* infructescences ($N=22$ species; 1–34 samples per species; 1–35 infructescences per sample; 0.45–63.49 g fresh weight; electronic supplementary material, data file S1). *Piper* species were identified based on morphological characteristics, and at least one sample per species was reviewed by an expert and confirmed via genetic markers (below). We considered infructescences ripe if they had fully formed individual fruits with seeds throughout infructescence length, were plump and soft when squeezed gently (although some species are not soft when ripe, e.g. *P. paulowniifolium*) and easily removed from the plant. In some cases (e.g. *P. sublineatum*, *P. conceptionis*), slight colour changes further allowed us to select ripe fruits.

We collected VOCs from infructescence ('fruit' hereafter) samples for approximately 20 h via dynamic headspace adsorption using a push–pull system and standardized methods [35], and subsequently identified and quantified putative VOCs through a gas chromatography-mass spectrometry (GC-MS) pipeline that included verification with alkane standards and Kovats indices. Contaminants (e.g. compounds present in empty bag controls, non-natural VOCs) and all VOCs that were present in fewer than five samples were removed from the dataset (see electronic supplementary materials for full protocol). We conducted calibration curves to quantify key VOCs in our sample, but note that isomers/enantiomeric composition of these VOCs will need to be verified further. Recorded VOC abundances correspond to both the VOC absorption on the matrix (during sample collection) and the ion fragmentation and MS detector sensitivity, and thus may not accurately reflect the quantities of all VOCs in the bouquet. We controlled VOC abundances by dividing them by the weight of the sample and hours of collection time. Using averages of these corrected values for each species, we then calculated: (1) total VOC emission—the sum of abundances across all VOCs, (2) number of VOCs in the scent bouquet, and (3) Shannon–Wiener index of VOC diversity. While the latter might overestimate the contribution of rare and underestimate the contribution of common VOCs, this might be advantageous given the abundance of rare yet potentially important VOCs in *Piper* fruit scents (see Results).

(b) Aim 2: characterize *Carollia* diet

To quantify *Piper* consumption by the three *Carollia* species in our study site, we deployed mist nets from 18.00 to 22.00 h along trails to capture bats during the rainy season (July and September–December 2015), when *Piper* fruit abundance and diversity is greater [36]. We collected faecal samples from 318 individuals (*C. perspicillata* $N=84$, *C. sowellii* $N=111$ and *C. castanea* $N=123$) by placing each bat in a cloth bag immediately after capture, and for a maximum of 2 h before release at the capture site. Upon collection, we allowed faecal pellets to dry in an air-conditioned room for 1–2 days prior to storage in 1.5 ml vials. We identified seed species in rehydrated faecal pellets under a dissecting microscope (Leica M125) via morphological comparisons with a seed reference library we compiled at La Selva (available upon request). If we could not identify the species of a particular seed, we classified it as a morphotype (e.g. *Piper* Type 1). We coded each plant species or morphotype as present/absent in each individual faecal sample and summed the number of positive samples for each *Piper* species within each *Carollia* species. To expand the temporal scope of the diet data to cover all months of the year, we combined our data (i.e. number of positive samples per *Piper* species for each *Carollia* species) with those from published studies that used similar methods to characterize *Carollia* diet at La Selva [37,38]. This entailed adding the reported total positive samples for each *Piper* species to our dataset, except for morphotypes or unidentified

samples. Finally, we calculated the proportion in the diet of each *Piper* species for each *Carollia* species as $\text{Proportion}_{\text{Piper}X} = \Sigma (\text{positive } \text{Piper}X \text{ samples}) / \text{total number of samples}$.

(c) Aim 3: determine *Carollia* VOC preferences

To test bat preferences for specific VOCs, we conducted two-choice behavioural experiments on naive *C. castanea* inside a flight cage (Coleman, $3.048 \times 3.048 \times 2.1336$ m) under natural ambient conditions at La Selva in August 2017 (41 adult males and non-lactating, non-pregnant females; $N = 25$ positive trials). During each experiment, we recorded a bat's foraging behaviour (infrared-sensitive handycam 4 K HD video recording, Sony, Japan) and echolocation calls (condenser microphone CM16, CMPA preamplifier unit, Avisoft Bioacoustics, Berlin, Germany) as they responded to experimental samples. These consisted of unripe *P. sancti-felices* fruit (control) or unripe *P. sancti-felices* fruit with either 2-heptanol, α -caryophyllene or α -phellandrene in the same concentrations and emission rates they are found in ripe fruit. These VOCs were selected because they are abundant and common in ripe fruit scent across all the *Piper* species studied (α -caryophyllene and α -phellandrene), or relatively rare but characteristic of *P. sancti-felices* ripe fruit scent (2-heptanol; [24]; electronic supplementary material, data file S1). Samples were placed on 50 ml Falcon tubes, 40 cm apart, on a custom-built platform (see electronic supplementary materials for details). During each trial, we presented a bat with a choice between a control and an enriched sample, recorded for a maximum of 20 min per trial and five trials per bat, with new trials being conditional on a positive response on the previous trial. We randomized both the trial order and position (left, right) of the target choices between consecutive trials to minimize spatial learning effects. In the laboratory, we watched videos of the behavioural trials and recorded the individual's choice, defining target selection as a bat landing on a target and attempting to bite it. All individuals were released near the site of capture after experiments and processing were completed.

(d) Aim 4: link *Carollia* diet and VOC preferences to *Piper* VOC evolution

To generate a molecular phylogeny for statistical analyses (below) and corroborate field species identifications, we extracted and amplified one chloroplast (approx. 887 bp *petA-psbJ* region) and one nuclear (approx. 708 bp *internal transcribed spacer-2*, *ITS*) gene from leaf samples collected from each *Piper* plant used for VOC sampling. We inferred maximum-likelihood trees for individual markers using the GTR + I + G model in GARLI 0.951, and used the ITS gene tree, previously published divergence times [39–41], and the R 'chronos' [42] function to infer an ultrametric, time-calibrated phylogeny. See the electronic supplementary materials for full methods.

To summarize the ripe fruit VOC abundance dataset into fewer variables, we used the 'capscale' function from the R package vegan [43] to run a constrained, redundancy (multidimensional scaling, MDS) analysis based on Bray–Curtis distances. This generated 21 MDS axes with no negative eigenvalues (electronic supplementary material, table S2). Then, to identify which compounds explained most of the variation across these axes, we used the 'envfit' function [43] with 1000 permutations to fit VOC vectors onto the first five axes of the ordination. Of these, we selected those at $\alpha \leq 0.001$. We used the *Piper* phylogeny (above) and functions from the R packages mvMORPH [44] and geomorph [45] to determine whether the MDS scores showed significant phylogenetic signal, and to fit three models of continuous trait evolution: (i) Brownian motion (BM)—a random walk evolutionary process with a constant rate, (ii) Ornstein–Uhlenbeck (OU)—random walk evolution with

selection towards an optimum value, and (iii) early burst (EB)—rates vary and are highest towards the root.

To investigate reciprocal relationships between *Piper* VOC traits and *Carollia* diet, we adopted a hierarchical Bayesian approach in brms [46], which implements models in the Stan language [47]. Typical analyses comprise regressions, but both VOC traits and *Carollia* consumption are measured with error, therefore we modelled all variables as responses, each with an error structure influenced by the phylogeny. While *Carollia* consumption for each species—a proportion—was modelled as zero-enriched beta distributed variable, *Piper* VOC traits were modelled as normally distributed variables except for the Poisson-distributed variables VOC number and plant abundance. Based on Salazar *et al.* [48], the latter was incorporated into models to determine the relationship between differential availability of *Piper* species and bat consumption. Our approach resulted in 17 multi-response models, each including the consumption for each *Carollia* species and one other variable. Variables spanning a wide scale (e.g. individual VOC concentrations) had to be log-transformed and/or required the addition of a constant due to many zeros. Although this can change model fit depending on the constant selected [49], model fits using leave-one-out cross validations revealed no change in fit across constants spanning three orders of magnitude (0.1, 1, and 10). Each model ran for 25–200 K iterations, with the first 2–150 K iterations discarded as burn-in. Convergence was evaluated using the effective sampling size for both species-specific and sample-wide effects. See the electronic supplementary materials for more model details.

To detect adaptive shifts in the evolution of *Piper* fruit scent chemistry, we used the function 'l1ou' [50] on six datasets: (1) VOC MDS1–5 scores (greater than 50% variation); (2) $(\log_{10}(x + 1))$ abundance of the six 'major' VOCs explaining variation across MDS1–5 (figure 1; germacrene-D, *p*-cymene, β -myrcene, α -phellandrene, β -phellandrene 2-nonanone), (3) $(\log_{10}(x + 1))$ abundance of the three 'key' VOCs significantly associated with *Carollia*'s diet and/or preferred by bats (2-nonanone, 2-heptanol, α -caryophyllene), (4) total VOC emission, (5) number of VOCs, and (6) VOC Shannon–Wiener index. We used the phylogenetic Bayesian information criterion to evaluate shifts in the evolution of VOCs and MDS scores. Since the number of axes was large compared to the number of species, we simulated evolutionary histories with no adaptive shifts to test for the detected number of shifts occurring by chance under a constant evolutionary process. We simulated histories both under BM with trait covariance and under bounded BM without trait covariance to account for the presence of 0 values in the VOC dataset. For both, 100 simulated datasets were generated; l1ou was applied to each, and the number of shifts were compared to that detected in the VOC and MDS datasets. As the presence and absence of VOCs appeared to impact l1ou shifts, we inferred the evolutionary histories of major and key VOCs. To do so, we used the R function fitDiscrete from geiger [51] to model the evolution of presence/absence of the four VOCs based on an equal transition rates model ($0 \rightarrow 1 = 1 \rightarrow 0$ 'ER') and an asymmetric rates model (ARD). We used the Akaike information criterion with sample size correction (AICc), to identify the best-fit model, and determined which direction ($0 \rightarrow 1$, or $1 \rightarrow 0$) was faster. All code used in analyses is available in the electronic supplementary material, files and Github doi:10.5281/zenodo.5068213.

3. Results

(a) Aim 1: interspecific diversity in scent composition of *Piper* fruits

We identified 249 VOCs in ripe fruit scents across 22 sympatric *Piper* (electronic supplementary material, data file S1). Fruit

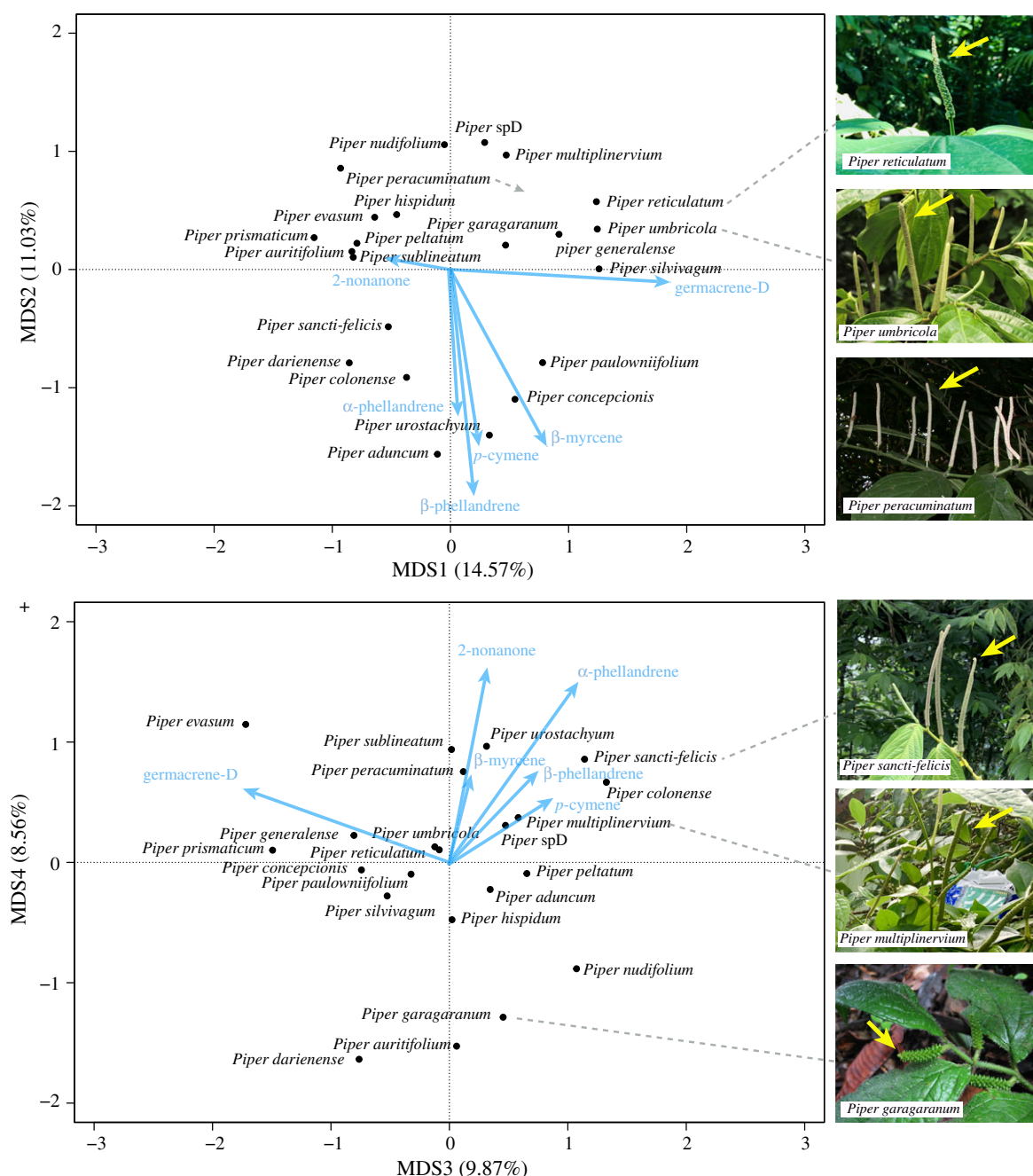


Figure 1. Distribution of the *Piper* species studied along the first four axes of an MDS analysis of ripe fruit VOC data. The presence and abundance of six major VOCs: 2-nonanone (ketone), germacrene-D (sesquiterpene), *p*-cymene (alkylbenzene), α -phellandrene, β -phellandrene and β -myrcene (monoterpenes) was significantly associated ($p < 0.0001$) with the first five axes of multivariate space of ripe fruit VOCs, as noted by the blue-coloured vectors. *Piper* species that are rare (*P. garagararum*) or predominant (all others) in the diet of *Carollia* are illustrated by photos on the right (fruits noted by yellow arrows). (Online version in colour.)

scents are dominated by sesquiterpenes (e.g. α -caryophyllene: 95% of species, germacrene-D: 77%, α -cubebene: 64%), monoterpenes (e.g. β -pinene: 64% of species, *trans*- β -ocimene: 59%, α -phellandrene: 55%, β -phellandrene: 45%), and to a much lesser extent, other compound types like aliphatic alcohols (e.g. 2-heptanol, 9% of species) and ketones (e.g. 2-nonanone, 14% of species). The number of identified VOCs in ripe fruit scent ranges from four (*Piper evasum*) to 104 (*Piper sancti-felices*) and varies widely in abundance across species (electronic supplementary material, data file S1). Out of the 21 multivariate axes of ripe fruit VOCs, MDS 1–5 explained 51% of the variance (electronic supplementary material, table S1). The presence and abundance of six compounds (major VOCs): 2-nonanone, germacrene-D, *p*-cymene, α -phellandrene, β -phellandrene and β -myrcene is significantly associated with MDS 1–5 ($p < 0.0001$; figure 1 and electronic supplementary

material, table S2, data file S2). Albeit a non-significant vector across these axes, α -caryophyllene strongly covaries with MDS1 (electronic supplementary material, data file S2).

Variation in fruit scent composition was not predicted by phylogeny (electronic supplementary material, figure S1; MDS1–21 $K_{\text{mult}} = 0.419$, $p = 0.859$; MDS1–5 $K_{\text{mult}} = 0.434$, $p = 0.143$), and neither was total VOC emission ($K = 0.751$; $p = 0.082$), nor the number ($K = 0.326$; $p = 0.797$) and diversity ($K = 0.433$; $p = 0.512$) of VOCs in ripe fruit scent across the *Piper* species studied.

(b) Aims 2 and 3: Bat diet and fruit scent preferences

The annual diet of the three *Carollia* species at La Selva is characterized by high abundance of a few and low abundance of many *Piper* species (figure 2 and electronic supplementary

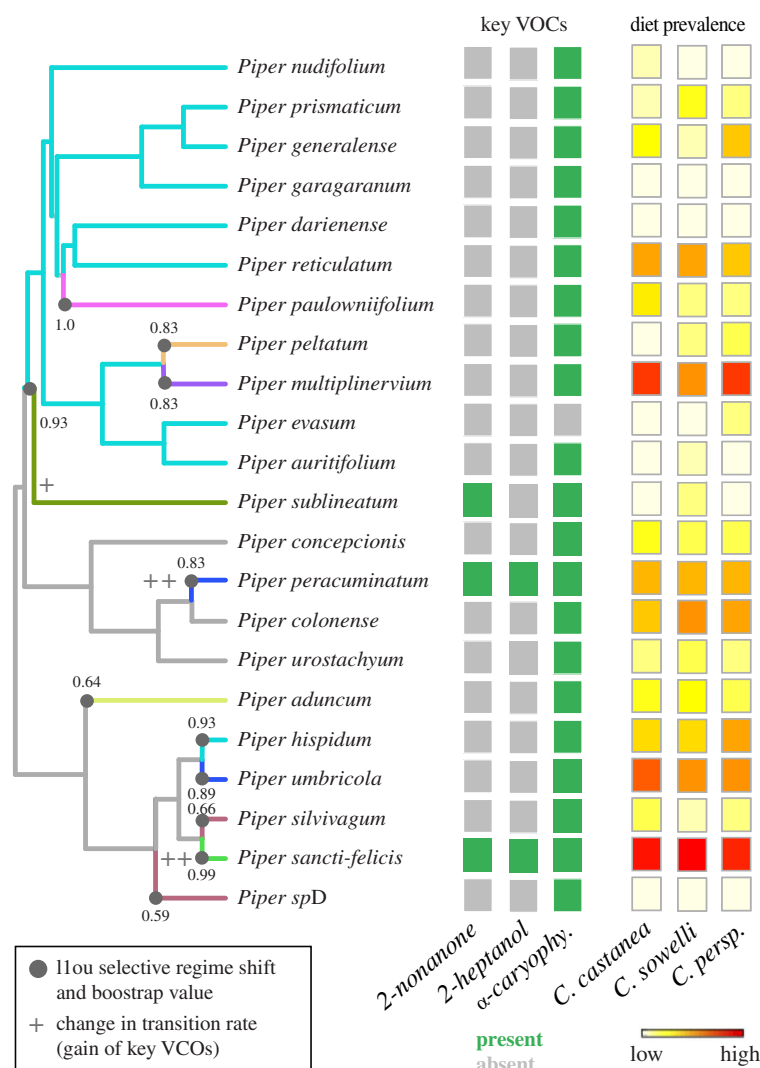


Figure 2. Main results of evolutionary analyses of fruit scent chemical composition. Phylogeny shows selective regime shifts (•) and associated bootstrap values from an 110 analysis of the six VOCs that significantly explain variation in fruit scent across *Piper* species (vectors in figure 1). Branches within each selective regime are noted in different colours. Also noted are nodes in which there were gains (+) of some key VOCs (i.e. those associated with diet, and/or preferred by bats), resulting in significant changes in evolutionary transition rates (table 2). The presence of the key VOCs is mapped on the tips of the phylogeny, along with the prevalence of each *Piper* species in the diet of each *Carollia* species. See Results for more details. (Online version in colour.)

material, table S3). *Piper sancti-felices* (*P. scintillans* [34]) has the greatest proportion of positive samples in the annual diet of all three *Carollia* (*C. castanea*: 28.63% of samples, *C. sowellii*: 35.44%, *C. perspicillata*: 23.38%). *Carollia castanea* has the greatest overall proportion of *Piper* species in its diet (79.05%), followed by *C. sowellii* (65.28%) and *C. perspicillata* (51.83%). Results of behavioural experiments suggest that bats prefer samples enriched with 2-heptanol (87.5% versus 12.5% control, $N=8$) and α -caryophyllene (75% versus 25% control, $N=8$), but show a neutral response to α -phellandrene (44.4% versus 55.6% control, $N=9$) when compared to solvent-only (unscented mineral oil) controls.

(c) Aim 4: links among *Carollia* diet, VOC preferences and *Piper* VOC evolution

Table 1 summarizes the results from multi-response models relating plant and fruit scent variables with the proportions of *Piper* species in the annual diet of each *Carollia* species (electronic supplementary material, figures S2–S6 show additional model results). These revealed no association between *Piper* plant abundances and the diet of any of the *Carollia* species.

There is a statistically positive association between MDS3 and the diet of *C. sowellii* and *C. perspicillata*, with a positive trend for *C. castanea*, as well as MDS4 and the diet of *C. castanea* and *C. sowellii*, with a positive trend in *C. perspicillata*. Total fruit VOC emission was not associated with the proportion of *Piper* species in the diets of any *Carollia* species. By contrast, the number of VOCs in ripe fruit scent is consistently, positively associated with the proportion of *Piper* in the diet of all three *Carollia*. VOC diversity in fruit scent is positively associated with the proportion of *Piper* species in the diet of *C. castanea* with positive trends for *C. sowellii* and *C. perspicillata*. We also found statistically supported associations between the abundance of 2-nonanone (one of the major VOCs) and the proportion of *Piper* species in the annual diet of *C. castanea*. Among the VOCs used in preference trials, we found a positive association between the abundance of 2-heptanol in ripe fruit scent and the proportion of *Piper* species in the annual diets of *C. castanea* and *C. sowellii* (with a positive trend in *C. perspicillata*), but no association with α -caryophyllene or α -phellandrene. Model fits from leave-one-out posterior predictive checks revealed some data points led to better model fit, therefore some models were not robust to subsampling.

Table 1. Posterior estimates of the coefficient of plant traits on *Carollia* consumption, *italics*: 95% credible intervals do not overlap with 0.

plant/fruit scent trait	<i>C. castanea</i>	<i>C. sowelli</i>	<i>C. perspicillata</i>
plant abundance	0 (−0.01–0.01)	0 (−0.01–0.01)	0 (−0.001–0.01)
MDS1	−0.02 (−0.76–0.69)	−0.14 (−0.88–0.45)	−0.05 (−0.70–0.52)
MDS2	0.15 (−0.63–0.82)	0.15 (−0.51–0.88)	0.39 (−0.29–1.14)
MDS3	0.81 (−0.12–1.90)	1.22 (0.14–2.25)	0.90 (0.09–1.89)
MDS4	1.95 (0.38–3.42)	1.59 (0.01–3.17)	0.77 (−0.63–2.52)
MDS5	0.71 (−0.59–2.19)	0.52 (−0.28–1.87)	0.47 (−0.43–1.59)
total emission	0.08 (−0.94–0.98)	−0.05 (−0.83–0.71)	−0.03 (−0.86–0.70)
VOC number	0.03 (0.01–0.05)	0.04 (0.02–0.05)	0.03 (0–0.05)
VOC diversity	1.48 (0.04–2.93)	1.35 (−0.10–2.81)	0.82 (−0.33–2.13)
2-heptanol ^a	0.40 (0.06–0.72)	0.50 (0.18–0.77)	0.35 (−0.01–0.64)
2-nonanone	0.65 (0–1.19)	0.35 (−0.24–1.08)	0.55 (−0.03–1.05)
germacrene-D	0.07 (−0.31–0.39)	−0.01 (−0.34–0.29)	0.02 (−0.26–0.34)
<i>p</i> -cymene	0.02 (−0.28–0.46)	0.07 (−0.21–0.46)	−0.03 (−0.26–0.23)
α -caryophyllene ^a	0.09 (−1.41–1.08)	0.07 (−0.84–0.87)	0.17 (−0.23–0.67)
α -phellandrene	0.12 (−0.22–0.46)	0.15 (−0.11–0.44)	0.10 (−0.18–0.47)
β -myrcene	−0.05 (−0.41–0.30)	−0.05 (−0.41–0.28)	−0.19 (−0.61–0.12)
β -phellandrene	0.29 (−0.11–0.75)	0.29 (−0.09–0.84)	0.13 (−0.18–0.60)

^aPreferred by bats.

A BM (non-adaptive) model was supported over a single selective peak (adaptive; OU) model for total VOC emission and MDS1–5 axes of ripe fruit scent composition (electronic supplementary material, tables S4–S5). While an adaptive model with three selective regime shifts was detected for MDS1–5, this result was highly likely when data were simulated under BM with trait covariance ($p=0.77$; electronic supplementary material, figure S7). By contrast, adaptive (OU) models received the highest support for the number and diversity of VOCs in fruit scent (electronic supplementary material, table S5), with two shifts leading to low and high VOC number and diversity in *P. evasum* and *P. sancti-felices*, respectively ($p=0.07$; electronic supplementary material, figure S8). For the six major VOCs, which significantly explained MDS1–5 variation, IIO analyses identified 11 selective regime shifts (three with greater than 90% bootstrap support, figure 2 and electronic supplementary material, figure S9). Simulations indicate that finding this number of shifts is highly unlikely when traits are simulated under BM with covariance ($p<0.01$), or as non-covarying bounded values ($p<0.01$). One well-supported, earlier shift at the split between *P. sublineatum* and a multispecies clade seems to be defined by the loss of β -myrcene, α/β -phellandrene and *p*-cymene. More recent shifts lead to clades represented by single species in our phylogeny (figure 2), with those basal to *P. hispidum* (93% bootstrap) and *P. sancti-felices* showing greatest support (greater than 92% bootstrap). For the three key VOCs linked to diet and/or preferred by *Carollia*, IIO identified three shifts leading to single species (electronic supplementary material, figure S10). Finding these many shifts was likely when traits were modelled under BM ($p=0.61$) or as non-covarying bounded values ($p=0.48$). The absence of 2-heptanol and 2-nonanone in most species was largely responsible for these results. Analyses of VOC data as presence–absence characters indicated transition

rates significantly differ for 2-heptanol and 2-nonanone: both had fewer gains than losses (table 2 and electronic supplementary material, figure S11).

4. Discussion

Ecological interactions can influence trait evolution, but the evolutionary history of organisms can also influence species associations and explain the variation in intervening phenotypic traits. While secondary metabolites that constitute ripe fruit scent are assumed to evolve adaptively to mediate interactions with frugivores, empirical evidence is scant. Using comparative phylogenetic analyses and Bayesian modelling, we show *Piper* phylogenetic relationships fail to explain fruit scent composition in sympatric *Piper* species, consistent with patterns previously found for other *Piper* chemical traits [48]. Instead, some components of *Piper* ripe fruit scent vary in tandem with their ecological relationship with their main dispersers within an ecological community. At our study site, *Carollia* heavily rely on *Piper* as a food resource and locate fruit using scent and specific VOCs. These, in turn, evolved and diversified directionally in *Piper* species highly consumed by *Carollia*.

Despite diverse influences on the variation and evolution of different components of ripe fruit scent and incomplete phylogenies, we find evidence of adaptive evolution in *Piper* scent. The *Piper* species analysed spanned major neotropical clades and high variance in chemical richness, reducing the influence of missing taxa with correlated traits [52] that might affect tests of phylogenetic signal and evolutionary model fitting [53]. Total VOC emission and MDS axes evolved away from the ancestral state in a diversifying pattern, perhaps reflecting the variety of habitats and physiologies of the *Piper* species studied, as secondary metabolite production,

Table 2. Results of fitDiscrete analysis of the rates of presence–absence transitions of eight key VOCs. Italic font indicates most supported model for each VOC. Note the enantiomeric composition of some of the monoterpenes will need to further verified.

VOC	equal rates model			all rates different model			transition rates	
	loglik	AIC	ΔAIC	loglik	AIC	ΔAIC	0–1	1–0
2-heptanol ^{a,b}	–9.51	21.22	3.19	–6.70	18.04	0	0.39	3.95
2-nonanone ^a	–11.03	24.27	2.11	–8.76	22.16	0	0.50	3.15
germacrene-D	–11.65	25.50	0	–11.35	27.34	1.84	0.03	0.01
<i>p</i> -cymene	–15.12	32.43	0	–15.11	34.85	2.42	0.03	0.03
α-caryophyllene ^b	–4.69	11.57	0	–4.05	12.73	1.16	0.09	0.004
α-phellandrene	–14.55	31.31	0	–14.29	33.22	1.91	<0.0001	0.01
β-myrcene	–13.95	30.09	0	–13.80	32.23	2.14	0.004	0.01
β-phellandrene	–15.25	32.70	0	–15.16	34.95	2.25	1.81	2.17

These chemicals explain variation in ripe fruit scent across *Piper* (figure 1), are significantly associated with *Carollia*'s diet (^a; table 1), and/or are preferred by *Carollia* (^b).

including VOC emission, responds to abiotic factors such as nutrient availability [54,55]. By contrast, VOC number and diversity fit adaptive models with selective peak shifts leading to single species. While most *Piper* species may experience stabilizing selection to maintain the ancestral optimum (i.e. a general 'Piper scent'), natural selection appears to have operated on specific clades to alter scent composition mainly by increasing or decreasing VOC number and diversity. Selective regime shifts detected for specific VOCs, some of which we demonstrate are directly and/or positively involved in interactions with *Carollia*, further bolsters evidence for VOC adaptive evolution.

We find many well-supported shifts in selective optima among the VOCs that drive fruit scent variation across the *Piper* species studied. Except for 2-nonanone, nearly all these compounds are terpenes or terpene-related chemicals (such as *p*-cymene). Terpenoids are some of the most abundant angiosperm VOCs, can serve as signals of fruit ripeness [28], and participate in plant defenses against herbivores and pathogens [32]. Therefore, a combination of signalling and defensive functions likely shaped the evolution of these chemicals in *Piper*. Minor structural changes in terpene synthases (TPS), a family of enzymes responsible for terpenes synthesis [56], can lead to major changes in product profiles. Molecular evidence also suggests that *de novo* synthesis of VOCs is induced at ripening, including transcriptional regulation of terpene-, carotenoid-, fatty acid- and phenylpropanoid-derived VOCs [56]. Thus, evolutionary differences in TPS structure, and the timing and degree to which different *Piper* species activate biosynthetic pathways during fruit ripening, could underlie the interspecific VOC patterns uncovered here.

Given their continuous or staggered phenologies [22], *Piper* provides *Carollia* with a constant supply of ripe fruit throughout the year, and *Carollia* primarily rely on *Piper* as a food resource in a manner inversely related to body size (Results; [23,57,58]). The prevalence of some *Piper* species in *Carollia*'s diet could be explained by their differences in number of fruits produced, seed number, or phenology, among other factors. However, behavioural studies conducted at La Selva have described *Piper* species preferences in *Carollia* that largely match trends in the diet data presented

here [38]. Importantly, the consumption of *Piper* species by *Carollia* was not statistically related to *Piper*'s reported abundances at our site [48]; bats select *Piper* fruit based on traits other than local abundance. Highly consumed *Piper* species are phylogenetically scattered and characterized by fruits scents rich in terpenoids, showing similarities with other bat-dispersed fruits (e.g. high abundance of monoterpenes; [26,28]). Major terpenoids, in turn, underwent multiple selective regime shifts (figure 2). If the differential consumption and subsequent dispersal by *Carollia* results in fitness benefits for *Piper* (as shown by [30,31]), then the dispersal syndrome hypothesis could explain evolutionary patterns in fruit scent chemistry, particularly for the VOCs preferred by and/or found in greater abundance in *Piper* species highly consumed by *Carollia*.

Bats likely use a complex hierarchy of signals to locate and select fruit (e.g. [24,59]). Although our VOC preference data are limited, the *Piper* specialist *C. castanea* tends to prefer samples rich in α-caryophyllene, a sesquiterpene found in the ripe fruit scent of almost all the *Piper* species studied (figure 2 and electronic supplementary material, figure S12), in greater amounts in species abundant in the diet of *C. castanea* (*P. multiplinervium*, *P. reticulatum*, *P. umbricola*), while also ubiquitous in angiosperms [60]. It is possible α-caryophyllene is part of the VOCs that change in abundance during the ripening process and together function as coarse signals for ripe *Piper* (e.g. in addition to α-phellandrene, which is not preferred by itself but abundant in some highly consumed *Piper* species). By contrast, 2-heptanol is preferred by and statistically associated with *Carollia* diet, and exhibited a significant change in evolutionary transition rate (gain) in *P. sancti-felices* and *P. peracuminatum*. *Piper sancti-felices* is an early succession tree that produces fruit year-round, thus the evolution of 2-heptanol in its fruit scent may constitute a feature that enhances bat attraction, for example, in the face of competition with other *Piper* for *Carollia*, or between *Carollia* and opportunistic but perhaps less efficient seed dispersers such as generalist birds (S.E.S. 2019 personal observation). As *P. peracuminatum*'s fruiting period is concentrated during the rainy season, when many other *Piper* also produce fruit, similar mechanisms could operate in this species to evolve 2-heptanol as a

bat signal. Lipid-derived precursors and VOCs become abundant as lipid biosynthesis and membrane fluidity increase during fruit ripening [61], thus 2-heptanol might be an indicator of fruit ripeness in those species in which it is present. Experimental evidence indicates *Carollia* has greater olfactory sensitivity to longer aliphatic alcohol C-chains [62], suggesting these bats may have specialized to perceive compounds such as 2-heptanol as a chemical signal.

Another fatty acid derivative, 2-nonanone, is significantly associated with *Carollia* consumption and underwent a change in evolutionary transition rate to be present in three species, *P. sancti-felices*, *P. peracuminatum* and *P. sublineatum*. While bat preferences for this compound are unknown, 2-nonanone is present in the fruit scent of other plant species (e.g. strawberries, raspberries [63], *Ficus* spp. [64]), in some of which it has antifungal properties [63]. Previous work has demonstrated that non-volatile antifungal compounds (amides) in *Piper* fruit have a deterrent effect on bat consumption [65]. Thus, the presence and positive association between 2-nonanone and *Carollia*'s consumption highlights other potential chemical strategies *Piper* species might have evolved to prevent fungal attacks while remaining attractive to bats.

Importantly, 2-nonanone and 2-heptanol are part of a highly diverse scent bouquet, particularly in *P. sancti-felices*, and VOC number and diversity are associated with *Carollia* diets. Fruit secondary metabolites may control multiple aspects of seed dispersal, such as frugivore seed removal timing and dispersal distances [66], and VOC number and diversity may also respond to abiotic and biotic factors that affect chemicals in other plant parts (e.g. leaves [48]). Therefore, other processes related to bat behaviour and physiology, and factors influencing secondary metabolite production, likely contributed to the diversification of *Piper* fruit scent VOCs described here.

5. Conclusion and future directions

We provide strong evidence for mediation of *Carollia*-*Piper* interactions by chemical signals, as traits involved in a likely dispersal syndrome in neotropical *Piper*. Yet, scent is one trait relevant to bat consumption; other plant and fruit characteristics may affect *Carollia*'s *Piper* consumption. For example, *P. garagaranum* (figure 1) has moderate amounts of α -caryophyllene in its fruit scent but is a small plant, so it may be difficult for bats to collect fruit. Future studies should integrate our findings with other aspects of *Piper* phenotype and ecology to flesh out coevolutionary dynamics between these plants and their seed dispersers. How VOCs are perceived by bats, and to what extent their responses to

scent are innate or learned also remain unknown. While the *C. perspicillata* olfactory receptor repertoire appears nondescript compared to those of non-frugivorous phyllostomids, *C. castanea* repertoires are distinctive even within the genus, likely contributing to *Piper* specialization [67]. This may have important consequences for the proposed coevolution with chemical fruit traits. As revealed by our analyses, the role of the *Carollia*-*Piper* mutualism on fruit scent evolution is complex and nuanced, making it an exciting system for future research.

Ethics. Research was conducted under Costa Rican permits SINAC-SEGUCUS-PI-R-117-2015, 038-2017-ACCPI, ACC-PI-030-2018, R-041-2016-OT-CONAGEBIO. All procedures for bat capture and handling were approved by the University of Washington's and Stony Brook University's Institutional Animal Care and Use Committees (#4307-02 at UW and #2013-2034 - R1- 8.15.19 BAT at SBU).

Data accessibility. All raw data and R code used for analyses can be found in the electronic supplementary materials file and in the Github repository <https://github.com/lmdavalos/mutualism>, doi:10.5281/zenodo.5068213.

The data are provided in electronic supplementary material [68].

Authors' contributions. S.E.S.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing-original draft, writing-review and editing; Z.A.K.: conceptualization, data curation, formal analysis, investigation, methodology, validation, writing-review and editing; L.B.L.: data curation, formal analysis, investigation, writing-review and editing; M.E.L.: investigation, writing-review and editing; J.A.: formal analysis, methodology, visualization, writing-review and editing; L.M.D.: conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, visualization, writing-review and editing; J.R.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, validation, writing-review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

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