Scents and Sense Ability:
The evolution and role of chemical cues and sensing in the pollination and herbivory of *Passiflora*

Marie Rose Clifford

A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2017

Reading Committee:

Jeffrey A. Riffell, Chair

Benjamin B. Kerr

Thomas L. Daniel

Program Authorized to Offer Degree:

Biology

University of Washington

Abstract

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Marie Rose Clifford

Chair of the Supervisory Committee:
Professor Jeffrey A. Riffell
Biology

Pollination and herbivory play a critical role in both wild ecosystems and agricultural ones, factoring in to their maintenance, evolution, and ecology. Insects, for whom chemical cues are often more important than those of other modalities, are primary drivers of both of these processes. We investigated the role and evolution of scent in the pollination and herbivory of *Passiflora*, a large genus of flowering plants for which relationships with pollinators and herbivores are comparatively well-documented. To understand these processes from the perspective of both plant and animal, we used integrative methods including phylogenetics, sensory electrophysiology, analytical chemistry, and machine learning to investigate.

On the pollination side, we find convergent evolution in floral morphology and floral scent, that these traits evolved in tandem to attract a given type of pollinator across *Passiflora*, and that these traits have the power to predict pollinator type. We further show that such floral scent changes may be biologically relevant to available pollinators using electrophysiological methods. Though future work is required to confirm the generality of this finding in additional plant and pollinator clades, this is a critical step to better understanding the role that chemical cues play in pollinator attraction, and the evolutionary synergy they may have with morphological traits in flowering plants.

In contrast, on the herbivory side, we find no relationship between herbivore and leaf scent. The chemical make-up of leaf scent is not explained by herbivore identity in *Passiflora* species. Furthermore, we do not find distinct sensory responses to leaf scents from host plants versus non-host plants in herbivorous insects. However, in exploratory work we do find some other factors that may explain patterns in herbivory, such as geographic distribution, which may warrant additional investigation in this system.

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ACKNOWLEDGEMENTS

"Gratitude is one of the least articulate emotions of the emotions, especially when it is deep."
– Felix Frankfurter

"It takes a village to raise a dissertation."
– Academic proverb

I am near overwhelmed with gratitude when I think about the support, kindness, and time generously given by so many, without which I would not have been able to complete this work.

To the funding organizations that gave me the means and opportunity to do research: the NSF, BEACON, Riddiford-Truman student grant, King Conservation District (UPP), US Fish and Wildlife (UPP), and our crowdfunding donors (UPP).

To my collaborators and advising scientists, who were unrelentingly generous with their time and expertise and who contributed significantly to the research in this dissertation and other projects: John MacDougal, Susan Waters, John Chau, Stephen Buchmann, and Larry Gilbert.

To my remarkable student-collaborators, who spent years with me collecting samples, doing experiments, and so much more: Nicholas Cutlip, Anakarina Lance, Nathan Wornian, Kaylie Lungberg, Jenny Eom, Ashley Stone, Eunice Kim, Tessa Forbes, and Olisavia Veliz.

To the UW Greenhouse staff, in particular Doug Ewing, who cared for my plants and also re-taught me over and over how and why to love the natural world: Jeanette Milne, Nile Kurashige, Paul Beeman, Terry Huang, Brian Watson, Keith Possee, and more.

To my advisor and committee, who gave me advice and guidance both personal and professional, and shaped me into an independent, resourceful researcher: Carl Bergstrom, Ben Kerr, Tom Daniel, Adrienne Fairhall, and Jeff Riffell.

To my labmates and a number of other grad students who have been a second family; teaching me to think, to fix machines and analyze data, to challenge and be challenged, and all kinds of other things, on top of being incredible friends: in particular, Yasmeen Hussain, Chloe Lahondere, Clement Vinauger, Kelsey Byers, Josh Nahum, Emily Grason, Susan Waters, Tracy Larson, Steve Springer, Kylee Petersen, Jay Stafstrom, Lauren Debey, and Biohouse – Daril Vilhena, Elisha Harris, Peter Conlin, Frazer Meacham, and Stephanie Crofts.

To UW Biology: Dave Hurley, Wai Pang Chan, Karen Bergeron, Marissa Heringer, and more.

To my teachers, who fostered this love of evolution, insects, science, and discovery in me: Ben Kerr, Evan Sugden, Victor Garcia, Jonna Dunham, and many more.

To my amazing friends and co-workers who have been accommodating, understanding, and incredibly supportive: Kyle Stanley, Brittany Lichty, Daril Vilhena, Brianna Mills, Molly Michal, Stephanie Kovacs, Andrew Hautau, Kyle Langford, Yi Chen, Anne Holm, Sam Mortimer, Laura Grit, and many, many more.

To my partner James Wilson and my family – my mom Alice Clifford, my dad Edwin Clifford, and my brothers Rob, Terrence, and Steve Clifford – who have believed in me unrelentingly and loved me unconditionally.

Thank you.

DEDICATION

To my dad, Edwin J. Clifford.

Who taught me,

How to work hard, love deeply, think critically, and joke badly,

And most importantly,

That success is born from treating people with kindness and respect.

I'll love you always.

Chapter 1. EVOLUTIONARY DIFFERENCES IN VISUAL VERSUS CHEMOSENSORY INVESTMENT IN THE CENTRAL NERVOUS SYSTEM OF SOCIAL INSECTS

Brain Size and Visual Environment Predict Species Differences in Paper Wasp Sensory Processing Brain Regions (Hymenoptera: Vespidae, Polistinae) (2013)

Original Paper

Brain, Behavior and Evolution

Brain Behav Evol 2013;82:177–184
DOI: 10.1159/000354968

Received: July 10, 2013
Accepted after first revision: August 8, 2013
Published online: October 29, 2013

Brain Size and Visual Environment Predict Species Differences in Paper Wasp Sensory Processing Brain Regions (Hymenoptera: Vespidae, Polistinae)

Sean O'Donnell[a] Marie R. Clifford[b] Sara DeLeon[a] Christopher Papa[a]
Nazaneen Zahedi[a] Susan J. Bulova[a]

[a]Department of Biodiversity, Earth and Environmental Science, Drexel University, Philadelphia, Pa., and
[b]Department of Biology, University of Washington, Seattle, Wash., USA

Key Words
Antennal lobe · Mosaic evolution · Mushroom body · Optic lobe · Paper wasp · Vespidae

Abstract
The mosaic brain evolution hypothesis predicts that the relative volumes of functionally distinct brain regions will vary independently and correlate with species' ecology. Paper wasp species (Hymenoptera: Vespidae, Polistinae) differ in light exposure: they construct open versus enclosed nests and one genus *(Apoica)* is nocturnal. We asked whether light environments were related to species differences in the size of antennal and optic processing brain tissues. Paper wasp brains have anatomically distinct peripheral and central regions that process antennal and optic sensory inputs. We measured the volumes of 4 sensory processing brain regions in paper wasp species from 13 Neotropical genera including open and enclosed nesters, and diurnal and nocturnal species. Species differed in sensory region volumes, but there was no evidence for trade-offs among sensory modalities. All sensory region volumes correlated with brain size. However, peripheral optic processing investment increased with brain size at a higher rate than peripheral antennal processing investment. Our data suggest that mosaic and concerted (size-constrained) brain evolution are not exclusive alternatives. When brain regions increase with brain size at different rates, these distinct allometries can allow for differential investment among sensory modalities. As predicted by mosaic evolution, species ecology was associated with some aspects of brain region investment. Nest architecture variation was not associated with brain investment differences, but the nocturnal genus *Apoica* had the largest antennal:optic volume ratio in its peripheral sensory lobes. Investment in central processing tissues was not related to nocturnality, a pattern also noted in mammals. The plasticity of neural connections in central regions may accommodate evolutionary shifts in input from the periphery with relatively minor changes in volume.

Introduction

The central nervous system (CNS), particularly the brain, lies at the interface between animal physiology and behavior. The CNS comprises some of the most expensive animal tissues in both production and maintenance costs, and these costs impose strong penalties for excess CNS tissue growth [Laughlin, 2001; Niven and Laughlin, 2008; Navarette et al., 2011]. Functional compartmentalization of brain tissues is a common if not universal feature of

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Sean O'Donnell
Department of Biodiversity, Earth and Environmental Science
Drexel University
3245 Chestnut St., Philadelphia, PA 19104 (USA)
E-Mail so356@drexel.edu

CNS architecture. Distinct cognitive processes are often performed by anatomically discrete brain regions. The mosaic brain evolution hypothesis links this functional compartmentalization to adaptive brain investment [Barton and Harvey, 2000; Smaers and Soligo, 2013]. Under mosaic brain evolution, the sizes of brain regions evolve independently in response to selective pressures on the cognitive demands they process [Chittka and Niven, 2009; Shultz and Dunbar, 2010]. Investment in brain regions should evolve rapidly and should closely match the particular cognitive demands animal species face. As predicted, comparative studies show that investments in brain regions covary with species' sensory environments [Cooper et al., 1993; Catania, 2005; Linsey et al., 2007]. For example, evolutionarily independent invasions of cave environments by *Astyamax* fish were followed by convergent but genetically distinct reductions of eyes and optic processing brain tissues [Borowsky, 2008; Jeffery, 2009]. Quantitative genetic and artificial selection studies demonstrate the feasibility of mosaic brain evolution: there are independent genetic effects on the sizes of different brain regions [Hager et al., 2012; Kolb et al., 2013].

A corollary of the mosaic brain hypothesis is the prediction of trade-offs (negative relationships) between the sizes of different brain regions: if functionally distinct regions are free to evolve independently, and overall CNS investment is limited, then brain regions may compete for limited resources [Niven and Laughlin, 2008]. Evidence of brain region trade-offs is often sought in CNS structures that process distinct sensory modalities, such as vision versus mechanosensation [Cooper et al., 1993; Catania, 2005]. Perception of different sensory modalities is typically accomplished by unique sensory structures, and this functional discretization is often echoed in central processing brain regions [Farris, 2008].

We tested predictions of mosaic brain evolution, including tests for trade-offs, using a comparative analysis of brain structure in Neotropical paper wasps (Vespidae, Polistinae). Paper wasps use powered flight for locomotion, and metabolic constraints on CNS investment may be especially great for flying animals. All paper wasps are eusocial, with obligate group living in nests where offspring are reared cooperatively. Sensory structures and brain anatomy are well characterized for social Hymenoptera, including paper wasps [Ehmer and Hoy, 2000; Gronenberg, 2001; O'Donnell et al., 2011]. All paper wasps have image-forming compound eyes and chemosensory/tactile antennae. Anatomically distinct peripheral lobes process optic and chemosensory input [optic and antennal lobes, respectively; Gronenberg, 2001], and these lobes innervate distinct regions of central processing neuropils called mushroom bodies [MB; Strausfeld et al., 1998; Fahrbach, 2006]. Optic lobes innervate the collar region of the MB calyx, and antennal lobes innervate the lip region of the MB calyx [Gronenberg, 1999].

Paper wasp genera differ in exposure to light levels. Some Neotropical genera build open-comb nests, while others construct enclosing nest envelopes or nest in dark cavities [Wenzel, 1991]. Wasps in the genus *Apoica* are the only nocturnally active Neotropical paper wasps [Greiner, 2006].

We used a phylogeny for the 19 currently recognized genera of paper wasps in the western hemisphere to account for potential effects of evolutionary relationships on brain structure (fig. 1) [Wenzel and Carpenter, 1994; Carpenter et al., 2000; Carpenter, 2004]. We measured the volumes of antennal processing regions (antennal lobes and MB lips) and optic processing regions (optic lobes and MB collars), and we used the total volume of all other brain structures (henceforth brain remainder) to control for variation in total brain size. Brain remainder volume was greater than all structures against which it was compared, making it a robust index of overall brain size. Using brain remainder as a brain size index avoids statistical confounds that result from including the structures being analyzed in a measure of total brain volume.

The evolution of nocturnal behavior is associated with changes in CNS investment across several taxa. Investment in optic processing tissue decreases, while investment in other modalities increases in derived nocturnal taxa [Barton et al., 1995; Catania, 2005]. In bats, the evolution of roosting in dark, sheltered sites is associated with the evolutionary loss of UV vision capacity [Xuan et al., 2012]. Based on these comparative studies of vertebrate brain investment, we expected paper wasp species with darker nest environments and nocturnal species to invest relatively more in chemosensory processing brain regions (antennal lobe and lip region of the MB calyx) and less in optic processing brain regions (optic lobe and collar region of the MB calyx) [Barton et al., 1995].

We first tested whether paper wasp genera differed in the volume of the four sensory processing regions. We then asked whether the volumes of the optic and antennal sensory processing regions were negatively correlated as expected if there was an investment trade-off. We examined evidence for trade-offs in two ways. First, we tested whether the absolute volumes of the optic and antennal processing regions were negatively correlated. Positive correlations between brain region volumes are expected if evolutionary changes in overall brain size affect all brain regions,

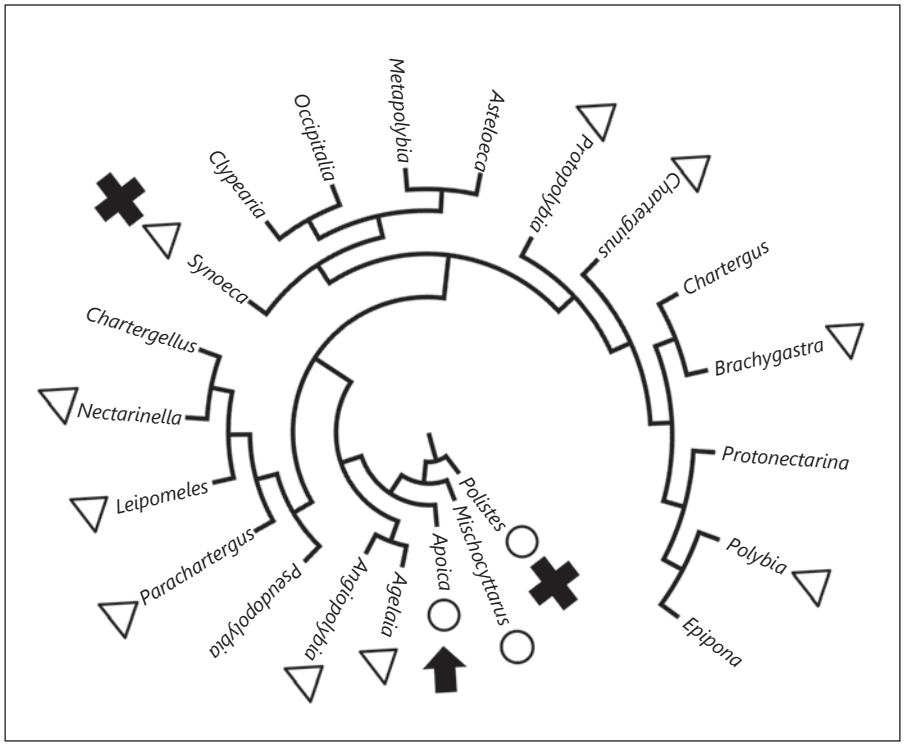


Fig. 1. Phylogeny of the 19 currently recognized Neotropical paper wasp genera [Wenzel and Carpenter, 1994; Carpenter et al., 2000; Carpenter, 2004]. The 13 subject genera are indicated with symbols as follows: circle = open nests; triangles = enclosed nests; arrow = nocturnal; plus sign = two largest-brained genera.

and this pattern could mask trade-offs among brain regions [concerted brain evolution: Herculano-Houzel, 2011; Powell and Leal, 2012]. To correct for possible concerted brain size effects, we also tested whether the ratios of volumes of each region to brain remainder were negatively correlated. As an alternative to direct trade-offs, we then tested whether overall brain size explained patterns of optic versus antennal brain investment. Investment in central versus peripheral processing brain regions depends on total brain size in paper wasps [O'Donnell et al., 2011]. Finally, to test whether brain region investment was associated with ecology (light levels), we asked whether nest architecture and nocturnal behavior predicted the size of the four sensory brain structures. We also used the ratios of the antennal to optic processing region volumes (antennal lobe/optic lobe and MB lip/collar) as indexes of relative tissue allocation among sensory modalities.

Materials and Methods

Subject Species

We analyzed the brain architecture of one species from each of 13 genera of Neotropical eusocial paper wasps (Polistinae). Our subjects spanned the 19 currently recognized Neotropical paper wasp genera, including relatively basal and derived taxa (fig. 1). All wasps were collected from nests in the field except *Brachygastra smithii* (collected from a swarm). Wasps were collected into and stored in buffered aldehyde-based fixative (Prefer fixative; Anatech, Ltd.). Wasp species, collection dates, and locations were: *Polistes instabilis:* July 2005, Costa Rica, 10°27.2′N, 85°7.5′W; *Mischocyttarus mastigophorus* and *Agelaia xanthopus:* August 2006, Costa Rica, 10°18.1′N, 84°47.9′W; *Nectarinella championi:* August 2006, Costa Rica, 10°14.4′N, 84°54.3′W; *Apoica pallens, Angiopolybia zischkai, Chartergunus fulvus, Leipomeles dorsata, Parachartergus smithii, Polybia dimidiata, Protopolybia exigua,* and *Synoeca septentrionalis:* June 2007, Ecuador, 0°40.3′S, 76°24.0′W, and *B. smithii:* July 2012, Costa Rica, 10°16.3′N, 84°49.4′W. We collected neuroanatomical data on 4–9 female wasps per species. Three subject genera build open-comb nests; the remainder construct enclosing envelopes or nest in cavities [Wenzel, 1991]. One genus *(Apoica)* is nocturnal [Greiner, 2006].

Histology and Neuroanatomy

Only mature wasps with fully hardened, deeply colored cuticle were used as subjects. We cut wasps' head capsules from the thorax at the narrow neck-like juncture behind the gena and removed the antennae and mandibles. We dehydrated head capsules through an ethanol series, acetone, then increasing concentrations of plastic resin. We incubated individual wasp heads in 0.5 ml resin in BEEM capsules at 60°C for 72 h. We sectioned each head into 12- to 16-μm-thick sections (depending on the species) using a rotary microtome with disposable steel histology blades. We mounted sections on gelatin-coated microscope slides and stained the tissue with toluidine blue. We cleared in an ethanol series and coverslipped under transparent mounting medium.

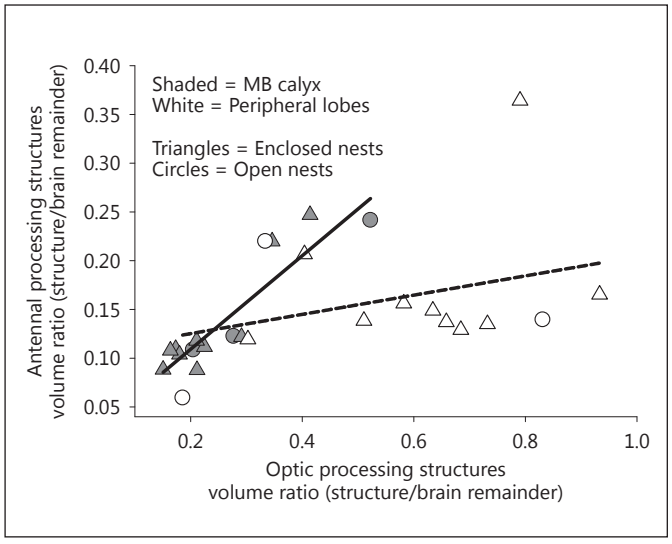


Fig. 2. Scatter plots showing correlations between brain size-corrected volumes of antennal and optic processing brain structures in the peripheral lobes and central brain (mushroom bodies). Values plotted are species means. Open vs. closed nests are indicated by symbols as noted. Least-squares linear regression lines are fitted for illustration purposes only in this graph; solid line = MB calyx data; dashed line = peripheral lobe data.

We used a microscope-mounted digital camera to photograph the tissue sections. For each wasp, we began photographing every other section at the section where brain tissue first became visible. We used ImageJ version 1.46 digital imaging analysis software (http://rsbweb.nih.gov/ij/) to estimate the volumes of brain structures. We outlined the target brain regions in the digital images and quantified the number of image pixels in the region, converting the pixel counts to area using a photograph of a stage micrometer as a size reference. Clear boundaries between the regions we measured were visible in all cases using our staining technique. We multiplied the areas by section thickness and distance between sections to yield volume estimates. We estimated the volumes of the following brain subregions: parts of the optic lobes (medulla and the lobula), the glomeruli of the antennal lobes, and the MB calyx lip and collar. We pooled other brain regions as an index of brain size: MB peduncle and lobes, the central complex, and the remainder of the protocerebrum, deutocerebrum, and tritocerebrum. We measured only brain neuropils; we did not measure adjacent cell body regions.

Statistical Analyses

All analyses were performed with SPSS v. 20 software (2011; IBM Corporation). We used Pearson's product moment to test correlations among region volumes. We used generalized linear models to test for relationships of ecological cofactors (nest architecture and nocturnality) with brain region sizes. We used ANCOVA to analyze the relationships of species means of MB calyx volume and peripheral lobe volume with the volume of remainder of the brain structures. The main effects terms of the ANCOVA tested whether the central and peripheral region volumes correlated with the size of the remainder of the brain. The (target region volume × brain remainder volume) interaction term tested whether the slopes of the two region-remainder correlations differed from each other.

For all analyses of species mean data, we conducted parallel analyses on phylogenetically independent contrasts (PIC) using the method of Felsenstein [1985] for two continuously varying characters. Independent contrasts were calculated using web-based software [COMPARE; Martins, 2004]. We calculated independent contrasts using a fully resolved genus level phylogeny for paper wasps [Wenzel and Carpenter, 1994] with all branch lengths set to one [O'Donnell et al., 2011]. In all cases, analyses of the raw data and PIC analyses led to similar conclusions.

Results

Species Differences

Species differed significantly in the relative amount of investment (ratio of brain region volume to brain remainder volume) for all four sensory processing brain regions (fig. 2; antennal lobes: $F_{12,74} = 24.87$, $p < 0.001$; optic lobes: $F_{12,74} = 35.45$, $p < 0.001$; MB lip: $F_{12,74} = 9.92$, $p < 0.001$; MB collar: $F_{12,74} = 11.27$, $p < 0.001$).

Tests for Trade-Offs

We found no evidence for direct trade-offs (negative relationships) between the sizes of paper wasp antennal and optic brain regions. For peripheral processing brain tissues, species mean antennal lobe volumes were significantly positively correlated with species mean optic lobe volumes ($r = 0.75$, $n = 13$, $p < 0.001$; PIC $r = 0.73$, $n = 12$, $p < 0.001$). For central processing brain tissues, species mean MB lip volumes were significantly positively correlated with species mean MB collar volumes ($r = 0.97$, $n = 13$, $p < 0.001$; PIC $r = 0.97$, $n = 12$, $p < 0.001$). Similar patterns held when analyzing brain size-corrected volumes [(fig. 2; peripheral lobes: $r = 0.92$, $n = 13$, $p < 0.001$, PIC $r = 0.93$, $n = 12$, $p < 0.001$; MB calyx: $r = 0.31$, $n = 13$, $p = 0.31$, PIC $r = 0.30$, $n = 12$, $p = 0.34$ (both not significant but positive)].

Relationships with Brain Size

Species mean total brain volumes ranged from 0.084 mm^3 *(Leipomeles)* to 0.51 mm^3 *(Polistes),* a 6-fold difference. Brain remainder volume ranged from 0.041 mm^3 *(Leipomeles)* to 0.22 mm^3 *(Polistes),* a 5.4-fold difference. Both antennal lobe and optic lobe volumes increased with brain remainder volume (fig. 3; $F_{1,22} = 26.49$, $p < 0.001$). Optic lobes were larger than antennal lobes (test for brain region main effects $F_{1,22} = 27.93$, $p < 0.001$) and increased in volume with brain remainder faster than antennal

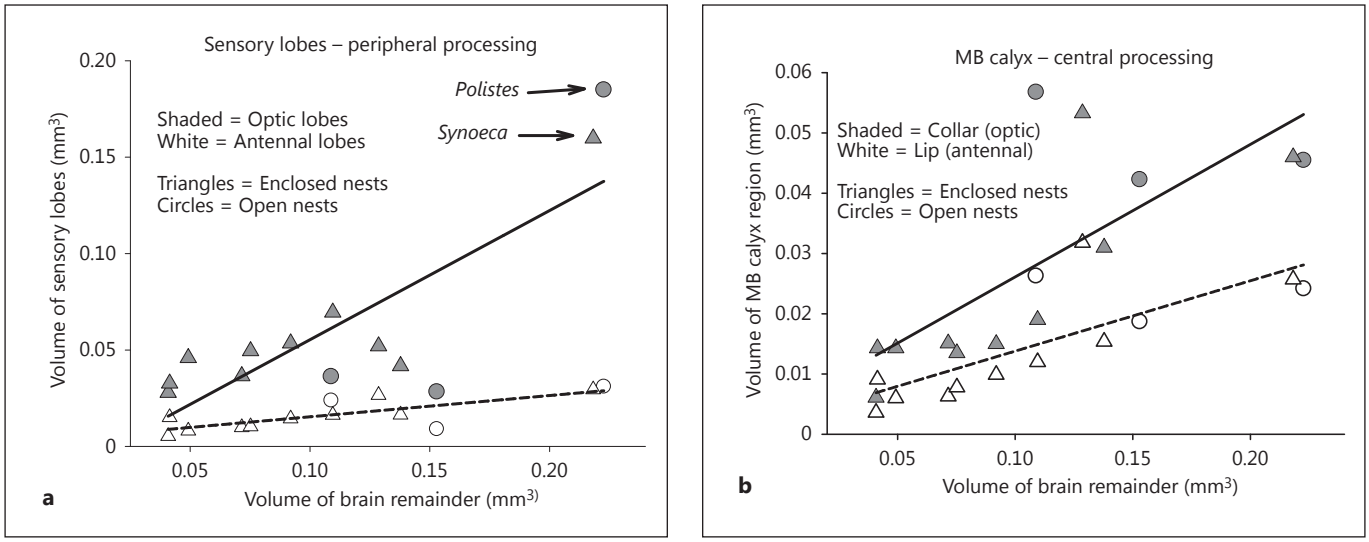


Fig. 3. Scatter plots showing the relationship of sensory structure volumes to brain size (brain remainder volume) in the sensory periphery (**a**) and in the central brain (**b**). **a** Data for the two largest-brained genera *(Polistes* and *Synoeca)* are indicated. Least-squares linear regression lines are fitted for each structure-size relationship; solid lines = optic structures; dashed lines = antennal structures.

lobes (test for differences in slope $F_{1,22} = 13.64$, $p = 0.001$; PIC ANCOVA $F_{1,20} = 10.10$, $p = 0.005$). The two largest-brained species had particularly large optic lobes (fig. 3). MB calyx lip and collar volumes both increased with brain remainder (fig. 3; $F_{1,22} = 25.52$, $p < 0.001$). MB collars were larger than MB lips (test for brain region main effects $F_{1,22} = 12.25$, $p = 0.002$), but these regions increased with brain remainder at similar rates [test for differences in slope $F_{1,22} = 2.40$, $p = 0.136$ (n.s.), PIC ANCOVA $F_{1,20} = 0.70$, $p = 0.41$].

Associations with Ecology: Light Levels

Species mean relative investment did not covary with nest architecture (open vs. closed nests) for any brain region (fig. 2, 3; antennal lobes: $F_{1,11} = 0.36$, $p = 0.55$; optic lobes: $F_{1,11} = 1.42$, $p = 0.26$; MB lip: $F_{1,11} = 0.45$, $p = 0.52$; MB collar: $F_{1,11} = 1.98$, $p = 0.19$). Nest architecture (open vs. closed nests) was not related to the antennal lobe:optic lobe ratio (fig. 3; $F_{1,11} = 1.22$, $p = 0.29$) or to the MB lip:collar ratio ($F_{1,11} = 2.23$, $p = 0.16$).

The evolution of nocturnality was associated with differences in sensory modality investment in the peripheral lobes. The nocturnal wasp *A. pallens* had the highest antennal lobe:optic lobe volume ratio among the species we studied (fig. 4; $F_{1,11} = 9.64$, $p = 0.010$), but this pattern was not reflected in the MB calyx lip:collar ratio ($F_{1,11} = 0.88$, $p = 0.37$).

Discussion

Paper wasp species differed in relative investments in the optic and antennal processing regions we measured, but increases in investment in one modality were not associated with decreases in investment in the other. Our data did not support the existence of direct trade-offs between sensory modalities. As seen in ants (Formicidae), volumes of optic and antennal regions were positively associated, suggesting concerted brain size effects on evolutionary changes in sensory structure volumes [Gronenberg and Hölldobler, 1999; Powell and Leal, 2012]. Evidence for direct evolutionary trade-offs between brain regions is rarely found [Barton and Harvey, 2000; Gatenby et al., 2011; Warren and Iglesias, 2012]. Many cases of apparent brain tissue trade-offs between sensory modalities involve taxa that have shifted to novel sensory environments such as caves or nocturnal activity [Zhao et al., 2009]. These cases may involve simultaneous selection for enhancing investment in one sensory capacity and selection against the other (or loss of function via neutral process such as drift). Recent suggestions that cave fish chemosensory tissue gain and optic tissue loss are genetically associated through negative pleiotropy have been called into question [Yoshizawa et al., 2012; Gunter and Meyer, 2013].

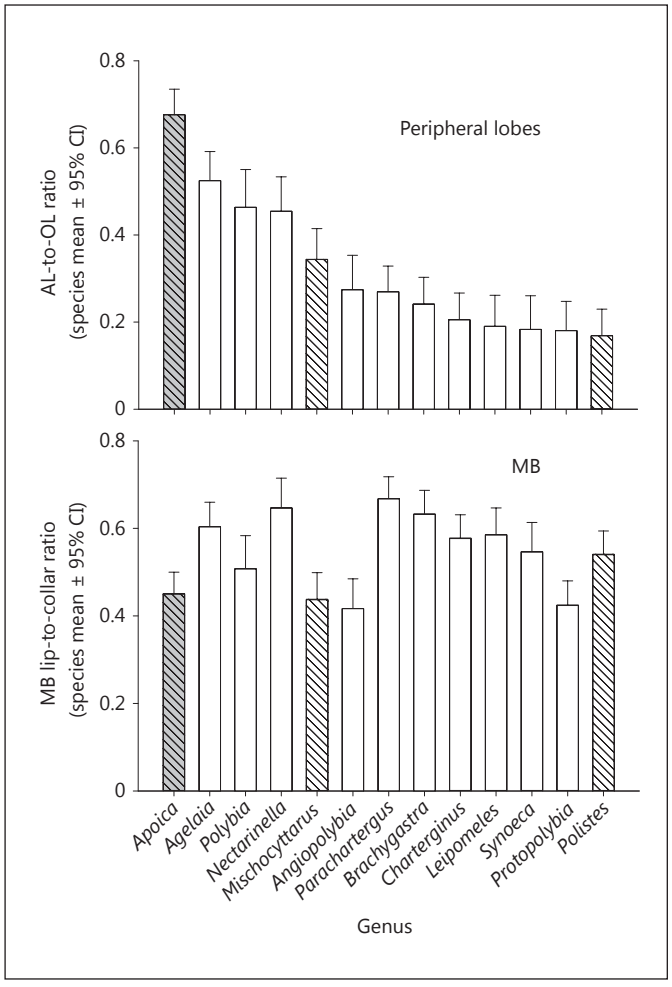


Fig. 4. Bar graphs showing the species mean values ± 95% CI (error bars) of the ratios of antennal:optic tissue volumes in the sensory periphery (top) and the central brain (bottom). Shaded bars are the nocturnal genus *Apoica;* hatched bars are species that build open-comb nests. Bars are ordered from species with the highest to lowest antennal lobe (AL):optic lobe (OL) ratio in both graphs.

Relationships with Brain Size

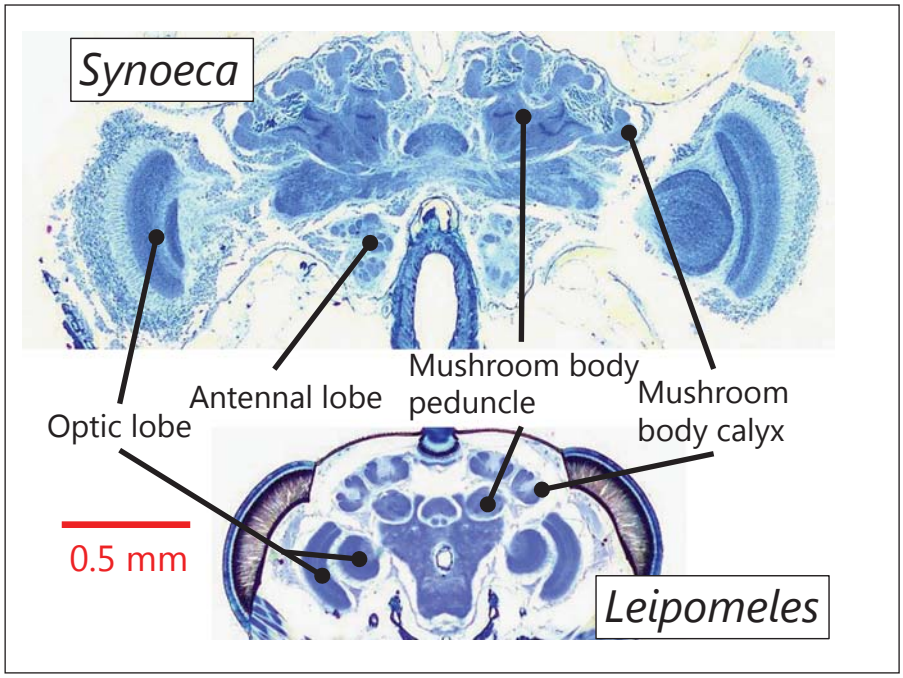
Paper wasp species differences in brain architecture were largely explained by brain size effects [also see O'Donnell et al., 2011]. Optic investment changed more rapidly with brain size than antennal investment in the peripheral sensory neuropils. Although a number of studies have attempted to test between concerted and mosaic brain evolution as alternatives [Barton et al., 1995; Herculano-Houzel, 2011; Powell and Leal, 2012; Gunter and Meyer, 2013; Smaers and Soligo, 2013], our findings suggest that this dichotomy is an oversimplification. Our data suggest that brain size is a key factor to be considered in analysis of brain architecture because brain regions that covary with brain size can do so at different rates [Kaskan et al., 2005]. While brain region investment may be constrained by overall brain size (concerted evolution), different rates of change with brain size can allow for evolutionary flexibility in tissue allocation to functionally distinct brain regions (mosaic evolution).

Both visual and optic sensory inputs are processed in hymenopteran brain regions we could not quantify due to limitations of our staining method. In honeybees *(Apis mellifera)* and other Hymenoptera, olfactory information is processed in the lateral horn region of the protocerebrum. Some axonal tracts that leave the antennal lobes innervate the lateral horn and are distinct from tracts innervating the MB lobes, suggesting parallel processing of sensory input in the lateral horn [Rössler and Zube, 2011]. Similarly, visual input is processed in the anterior optic tubercule as well as in the MB collar region [Pfeiffer and Kinoshita, 2012]. Quantification of the size of these other sensory processing regions could be used to test the generality of our findings.

Relationships with brain size differed between paper wasp antennal and optic peripheral lobes. The two largest-brained species had particularly large optic lobes (fig. 5). These species, i.e. *P. instabilis* and *S. septentrionalis,* differ from each other in the size of new and mature colonies and in nest architecture (open stalked combs vs. envelope-covered sessile combs), suggesting that there is a general effect of brain size on optic lobe investment in paper wasps.

Species Ecology: Visual Environments

Contrary to our predictions, paper wasp nest architecture variation was not associated with species differences in brain sensory structure sizes. In addition to lower light levels, the interiors of enclosed nests are likely to have relatively simple visual environments (e.g. less light intensity variation, fewer visual edges, less color variation). However, the relative darkness of wasp nest interiors may vary among species. Enclosed-nest subject species in our study ranged from cavity nesters *(A. xanthopus)* to species with thin, translucent nest paper *(L. dorsata).* Furthermore, swarm-founding wasp workers are often active on the exterior nest surface, and workers of all species must depart the nest to forage for food and other materials [O'Donnell and Jeanne, 1992]. These temporary exposures to exterior light environments may affect paper wasp brain investment in optic processing independently of nest visual environments.



Color version available online

Fig. 5. Light photomicrographs of stained histological sections along a frontal plane of two paper wasp subject species shown at the same scale. The sections shown are from the mid-region of the brain. Top: *S. septentrionalis* (largest-brained species); bottom: *L. dorsata* (smallest-brained species). Some of the neuroanatomical regions we quantified are indicated for each species. The scale bar refers to both photomicrographs.

The evolution of nocturnality in the genus *Apoica* led to dramatic changes in light levels experienced by active wasps. As predicted, the nocturnal paper wasp *Apoica* differed from other genera in its greater antennal lobe-to-optic lobe volume ratio. The evolution of nocturnality in flying Hymenoptera (bees and wasps) is associated with changes in compound eye structure: eye size (number of facets), light-gathering lens size, and/or the size of light receptive surfaces increase compared to diurnal relatives [Warrant et al., 2004; Greiner, 2006; Warrant, 2008]. However, these changes are not sufficient to maintain visual acuity under the eight-order-of-magnitude-lower light levels experienced during night flight, and neural processing such as summation of light inputs across eye facets may be necessary to permit nocturnal foraging [Greiner, 2006; Warrant, 2008; Kelber et al., 2011]. Our data suggest that, as in nocturnal vertebrates, the relative reliance on olfaction increases even when anatomical adaptations for low-light visual acuity arise. Nocturnal foraging evolved independently in the Paleotropical social hornet *Provespa* (Vespidae, subfamily Vespinae), providing an opportunity for additional comparative tests of the generality of our findings on sensory brain investment in wasps [Warrant, 2008].

The strongest brain architecture differences for *Apoica* were in peripheral sensory processing tissues, and these differences were not reflected in associated sensory processing regions of the MB. Similar evolutionary lability in the sensory periphery combines with central processing conservation in the evolution of novel sensory systems in vertebrates [Wilczynski, 1984; Kaskan et al., 2005]. Changes in patterns of innervation and neural connectivity in central processing regions such as vertebrate cortex and insect MB can accommodate developmental changes in environmental experience, individual differences in sensory structure neuron composition, and even loss of sensory input such as blindness [Kaskan et al., 2005; Jones et al., 2009; Merabet et al., 2010; Groh et al., 2012]. This plasticity may allow central processing tissues to accommodate evolutionary transitions in input from the sensory periphery without requiring proportional changes in the size of the central processing region.

Acknowledgements

An anonymous reviewer made valuable suggestions for improving this paper. Emily Johnson, Joshua Matlock, Yamile Molina, Abigal Mudd, Jason Stafstrom, Eve Swearingen, and Ceri Weber assisted with histology and neuroanatomy. Specimens were collected under research permits from the Republics of Ecuador and Costa Rica, and in accordance with their laws. M.R.C. was supported by an NSF Graduate Research Fellowship under grant No. DGE-0718124; S.O'D. was supported by NSF grant IOS-1209072.

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A test of neuroecological predictions using paperwasp caste differences in brain structure (Hymenoptera: Vespidae) (2014)

Behav Ecol Sociobiol (2014) 68:529–536
DOI 10.1007/s00265-013-1667-6

ORIGINAL PAPER

A test of neuroecological predictions using paperwasp caste differences in brain structure (Hymenoptera: Vespidae)

Sean O'Donnell · Marie R. Clifford · Susan J. Bulova · Sara DeLeon · Christopher Papa · Nazaneen Zahedi

Received: 31 July 2013 / Revised: 25 November 2013 / Accepted: 4 December 2013 / Published online: 19 December 2013

Abstract Adaptive brain architecture hypotheses predict brain region investment matches the cognitive and sensory demands an individual confronts. Social hymenopteran queen and worker castes differ categorically in behavior and physiology leading to divergent sensory experiences. Queens in mature colonies are largely nest-bound while workers depart nests to forage. We predicted social paperwasp castes would differ in tissue allocation among brain regions. We expected workers to invest relatively more than queens in neural tissues that process visual input. As predicted, we found workers invested more in visual relative to antennal processing than queens both in peripheral sensory lobes and in central processing brain regions (mushroom bodies). Although we did not measure individual brain development changes, our comparative data provide a preliminary test of mechanisms of caste differences. Paperwasp species differ in the degree of caste differentiation (monomorphic versus polymorphic castes) and in colony structure (independent- versus swarm-founding); these differences could correspond to the magnitude of caste brain divergence. If caste differences resulted from divergent developmental programs (experience-expectant brain growth), we predicted species with morphologically distinct queens, and/or swarm-founders, would show greater caste divergence of brain architecture. Alternatively, if adult experience affected brain plasticity (experience-dependent brain growth), we predicted independent-founding species would show greater caste divergence of brain architecture. Caste polymorphism was not related to the magnitude of queen-worker brain differences, and independent-founder caste brain differences were greater than swarm-founder caste differences. Greater caste separation in independent-founder brain structure suggests a role for adult experience in the development of caste-specific brain anatomy.

Keywords Antennal lobe · Brain evolution · Mushroom body · Neural plasticity · Optic lobe

Communicated by M. Giurfa

S. O'Donnell (✉) · S. J. Bulova · S. DeLeon · C. Papa · N. Zahedi
Department of Biodiversity, Earth & Environmental Science, Drexel University, 3245 Chestnut Street, Philadelphia, PA 19104, USA
e-mail: so356@drexel.edu

M. R. Clifford
Department of Biology, University of Washington, Seattle, WA 98195, USA

Introduction

Brain tissues are compartmentalized into anatomically discrete regions that perform distinct cognitive processes (Tanaka et al. 2012; Arrenberg and Driever 2013; Brown and Piscopo 2013; Mantini et al. 2013). Brain tissue is metabolically and developmentally expensive (Laughlin 2001; Niven and Laughlin 2008; Navarrete et al. 2011). Natural selection should act on brain architecture such that the relative investment in each brain region matches the animal's cognitive demands (Chittka and Niven 2009; Gronenberg and Riveros 2009; Shultz and Dunbar 2010). Investment in functionally distinct brain regions should reflect behavior and ecology at the individual and species levels (Cooper et al. 1993; Catania 2005; Linsey et al. 2007).

Social insect castes present an excellent opportunity to test adaptive brain architecture hypotheses. Queens and workers play distinct social roles, and neuroecological theory predicts their different behaviors and sensory experience will be reflected in distinct brain architectures (Gronenberg and Riveros 2009). In most social insects, reproductive castes are categorically distinct phenotypes that result from developmental plasticity (West-Eberhard 1981). In female eusocial Hymenoptera, reproductive queens and sterile workers can

develop from similar genotypes depending on the environment early in larval development (Hunt et al. 2007; Martins et al. 2010). We asked whether brain investment differed between reproductive castes—egg-laying queens and sterile workers—in eusocial paperwasps (Vespidae, Polistinae). Paperwasp queens and workers differ strongly in behavior and ecology. Queens are generally nest-bound, rarely flying from mature colonies, while workers perform diverse tasks including leaving the nest to forage (Herman et al. 2000; Bruyndonckx et al. 2006; Chavarria-Pizarro and West-Eberhard 2010; De Souza and Prezoto 2012). However, paperwasps vary widely in colony size and social complexity. Independent-founding species have relatively small colonies, and adult females are relatively plastic in their reproductive roles. Dominance interactions among females affect social status and opportunities for reproduction (Molina and O'Donnell 2008). Swarm-founding wasps have larger colonies with less plasticity in reproductive roles (Jeanne 2003). Paperwasp species further differ in the degree of female caste differentiation. Paperwasps range from species with no detectable caste categories (independent-founders and some swarm-founders) to swarm-founding species with discrete queen/worker body size and shape differences (O'Donnell 1998a; Noll et al. 2004).

We used the relative volumes of brain regions that process two distinct sensory inputs—the compound eyes (vision) and the antennae (chemosensation and tactile)—to test whether caste differences correspond to environmental context. Distinct brain regions process visual information from the compound eyes and chemosensory input from the antennae in social Hymenoptera, including paperwasps (Gronenberg 1999; Ehmer and Hoy 2000; O'Donnell et al. 2011). Anatomically distinct peripheral lobes process visual and chemosensory inputs (optic and antennal lobes, respectively; Gronenberg 1999; Hansson and Stensmyr 2011), and these lobes innervate distinct regions of central processing neuropils called mushroom bodies (Strausfeld et al. 1998; Fahrbach 2006). The optic lobes innervate the collar region of the mushroom body calyx, and the antennal lobes innervate the lip region of the mushroom body calyx (Gronenberg 1999). Both the antennal and optic lobes show structural plasticity following changes in sensory input and experience (Barth et al. 1997; Eickhoff et al. 2012; Arenas et al. 2012). Differences in mushroom body calyx volume are related to caste and task performance in many social Hymenoptera including paperwasps (Gronenberg et al. 1996; Farris et al. 2001; O'Donnell et al. 2004, 2007; Muscedere and Traniello 2012).

Because queens and workers occupy different sensory environments, and paperwasp brain regions vary in size with experience, we predicted there would be caste differences in relative investment in visual versus antennal processing brain regions. We used the ratio of visual processing to antennal processing tissue volume as an index of the degree of reliance on visual versus antennal inputs, both in the peripheral lobes and in the mushroom bodies. Paperwasp queens are largely nest-bound and experience less complex visual environments and lower light levels than their foraging workers. Behavioral activity under lower light conditions is associated with decreases in visual processing tissue (Catania 2005; Barton et al. 1995; Fujun et al. 2012). We predicted paperwasp workers would invest relatively more than their queens in visual processing brain regions (the optic lobes and the collar regions of the mushroom body calyx; Gronenberg 1999; O'Donnell et al. 2011).

Different developmental programs could lead to caste-specific brain architecture (experience-expectant brain growth). Alternatively, brain structure could respond to individuals' caste-specific environments (experience-dependent brain growth; Fahrbach et al. 1998; Farris et al. 2001). We used comparisons of paperwasp species with different social structures as an indirect means of assessing the relative importance of experience-expectant and experience-dependent brain development. We categorized our subject species as independent-founders (primitively eusocial) and swarm-founders (advanced eusocial), and we identified species with morphologically distinct queens (highly eusocial) (O'Donnell 1998a; Jeanne 2003; Noll et al. 2004). We tested whether the magnitude of queen-worker differences in brain architecture differed between the three social structure categories (independent-founders, caste monomorphic swarm-founders, and caste dimorphic swarm-founders). The degree of specialization of reproductive castes on different social roles increases as larger, more complex societies evolve from independent-founding ancestors with smaller colonies (Bourke 1999; Anderson and McShea 2001; Jeanne 2003). If experience-expectant growth predominates, we expected caste differences to be greater in species with morphologically distinct queen/worker castes and/or greater in swarm-founders relative to independent-founders. In contrast, reproductive caste status in independent-founders is relatively plastic and influenced by dominance interactions (Molina and O'Donnell 2008). If experience-dependent brain growth predominates, queen-worker differences could be stronger in independent-founders.

Materials and methods

Subject species

We analyzed the brain architecture of one species from each of 12 genera of Neotropical eusocial paperwasps (Polistinae). Our subjects spanned the 19 currently recognized Neotropical paperwasp genera, including the 2 relatively basal independent-founding genera (*Polistes*, *Mischocyttarus*) and 10 derived swarm-founding genera (Fig. 1; Wenzel and

Carpenter 1994; Carpenter et al. 2000; Carpenter 2004). Wasps were collected into and stored in a buffered aldehyde-based fixative (Prefer fixative, Anatech, Ltd.) for 1–5 years until histological processing. Subject species, collection dates, and locations were as follows: *Polistes instabilis*: July 2005, Costa Rica, 10°27.2′N, 85°7.5′W; *Mischocyttarus mastigophorus* and *Agelaia xanthopus*: August 2006, Costa Rica, 10°18.1′N, 84°47.9′W; *Nectarinella championi*: August 2006, Costa Rica, 10°14.4′N, 84°54.3′W; *Apoica pallens*, *Angiopolybia zischkai*, *Charterginus fulvus*, *Leipomeles dorsata*, *Parachartergus smithii*, *Polybia dimidiata*, and *Protopolybia exigua*: June 2007, Ecuador, 0°40.3′S, 76°24.0′W; *Brachygastra smithii*: July 2012, Costa Rica, 10°16.3′N, 84°49.4′W. All wasps were collected from nests in the field except *B. smithii* which were collected from a swarm. We categorized three species as having morphologically distinct reproductive castes based on published morphometric analyses and our observations (Shima et al. 1994, 1996; Hunt et al. 2001; Noll et al. 2004).

Determining subjects' caste

All subjects were mature wasps with fully hardened, deeply colored cuticles. We did not know the individual histories of the subjects and we assume our haphazardly chosen samples are representative of each caste. To determine the subjects' caste, we dissected their gasters (the terminal body region in aculeate Hymenoptera) in the fixative. We exposed the ovaries and examined them at ×10 magnification under a binocular dissecting scope. Workers had filamentous ovarioles with no visible opaque oocyte swellings. Queens had robust, well-developed ovaries with at least one fully opaque, oblong oocyte per ovariole. Individuals with intermediate ovaries were observed, but we only used the two extreme phenotypes as subjects for this study.

Histology and neuroanatomy

Histological processing was conducted in several bouts from September 2007 to September 2013. We collected neuroanatomical data on $n=82$ paperwasp subjects. We sampled four to nine wasps per species, with two to six individuals sampled from each caste (Table 1). We cut the fixed wasps' head capsules from the thorax at the narrow neck-like juncture behind the gena and removed the antennae and mandibles. Head capsules were dehydrated through a series of increasing ethanol concentrations, acetone, and then increasing concentrations of plastic resin (resin composition: 5.5 g of EMbed 812 (a mixture of bisphenol A/epichchlorohydrin epoxy resin (CAS #25068–38-6) and epoxy modifier (CAS #2425–79-8)), 5.7 g of dodecenyl succinic anhydride, 0.65 g of dibutyl phthalate, and 0.31 g of 2,4,6-tri(dimethylaminoethyl)phenol). We incubated individual wasp heads in 0.1-ml resin in pyramid molds at 60 °C for 72 h, then glued the resin to 0.5-ml acrylic cylinders with cyanoacrylate adhesive, and cut each head along the frontal plane into 12- to 16-μm-thick sections (depending on species) using a rotary microtome with disposable steel histology blades. Sections were mounted on gelatin-coated microscope slides, and the tissue was stained with toluidine blue. We cleared the stained sections in a series of increasing ethanol concentrations and cover-slipped under a transparent mounting medium.

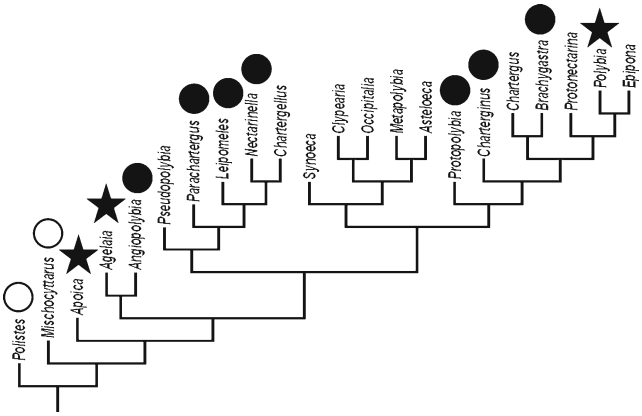

Fig. 1 Phylogeny of the 19 currently recognized subject paperwasp genera (Wenzel and Carpenter 1994; Carpenter et al. 2000; Carpenter 2004). The 12 subject genera are indicated with symbols as follows: *open circles*, independent-founding caste monomorphic species; *filled circles*, swarm-founding caste monomorphic species; *filled stars*, swarm-founding caste polymorphic species

We used a microscope-mounted digital camera to photograph the tissue sections at 2,560×1,920 pixel resolution, using ×2.5 or ×5 microscope objectives (depending on species). For each wasp, we began photographing every other section at the section where brain tissue first became visible. ImageJ version 1.46 digital imaging analysis software (http://rsbweb.nih.gov/ij/) was used to quantify the volumes of brain structures. We outlined the target brain regions and quantified the number of image pixels in the structure using ImageJ, and then we converted the pixel counts to area using a photograph of a stage micrometer taken at the same resolution and magnification as a size reference. We multiplied the areas by distance between sections to yield volume. We estimated the volumes of the following brain subregions: parts of the optic lobes (the medulla and the lobula), the glomeruli of the antennal lobes, and the mushroom body (MB) calyx lip and collar. Other brain regions were pooled into a volume referred to as brain remainder as an index of brain size: brain remainder volume=sum of estimated volumes of MB peduncle and lobes, central complex, and any protocerebrum, deutocerebrum, and tritocerebrum not included in the brain regions named above. We measured only brain neuropils (regions of dendritic arborization and axonal connections); we did not measure adjacent regions containing the cell bodies (somata) of the brain's intrinsic neurons. For analyses of caste

Table 1 Mean estimated volumes (mm^3) of brain regions for queen and worker castes from 12 species of social paperwasps

Species	Caste	Caste sample size	Mean MB collar	Mean MB lip	Mean OL	Mean AL	Mean remainder	Mean total
Agelaia xanthopus	Queens	4	0.047	0.030	0.043	0.024	0.111	0.255
Agelaia xanthopus	Workers	3	0.061	0.035	0.064	0.030	0.152	0.343
Angiopolybia pallens	Queens	2	0.016	0.008	0.041	0.011	0.074	0.149
Angiopolybia pallens	Workers	3	0.015	0.005	0.033	0.009	0.070	0.133
Apoica pallens	Queens	5	0.055	0.025	0.032	0.023	0.097	0.232
Apoica pallens	Workers	4	0.060	0.028	0.042	0.026	0.123	0.278
Brachygastra smithii	Queens	3	0.020	0.012	0.076	0.016	0.111	0.235
Brachygastra smithii	Workers	5	0.019	0.012	0.065	0.016	0.109	0.221
Charterginus fulvus	Queens	3	0.018	0.010	0.050	0.011	0.078	0.166
Charterginus fulvus	Workers	5	0.011	0.006	0.049	0.010	0.074	0.150
Leipomeles dorsata	Queens	3	0.007	0.004	0.027	0.005	0.040	0.082
Leipomeles dorsata	Workers	3	0.006	0.003	0.029	0.005	0.041	0.085
Mischocyttarus mastigophorus	Queens	3	0.046	0.022	0.026	0.010	0.147	0.250
Mischocyttarus mastigophorus	Workers	3	0.039	0.015	0.031	0.008	0.159	0.252
Nectarinella championi	Queens	2	0.017	0.011	0.031	0.014	0.040	0.114
Nectarinella championi	Workers	3	0.012	0.008	0.034	0.016	0.042	0.112
Parachartergus smithii	Queens	4	0.018	0.011	0.051	0.013	0.091	0.184
Parachartergus smithii	Workers	5	0.013	0.009	0.056	0.015	0.093	0.185
Polistes instabilis	Queens	2	0.038	0.027	0.142	0.028	0.225	0.459
Polistes instabilis	Workers	6	0.048	0.023	0.199	0.032	0.222	0.525
Polybia dimidiata	Queens	2	0.025	0.015	0.029	0.016	0.124	0.209
Polybia dimidiata	Workers	2	0.037	0.016	0.054	0.017	0.152	0.275
Protopolybia exigua	Queens	4	0.015	0.006	0.043	0.008	0.049	0.121
Protopolybia exigua	Workers	3	0.014	0.006	0.050	0.008	0.050	0.127

MB mushroom body, *OL* optic lobe, *AL* antennal lobe, *Remainder* brain remainder, sum of other brain structures, *Total* total volume of all brain structures measured

differences in the volumes of single structures, we used brain size-corrected volumes: structure volume/brain remainder.

Analysis of caste differences

We used general linear models to analyze the data (SPSS v. 20, IBM Corp., 2011). We developed multivariate models to test relationships of response variables (brain region volume ratios or brain size-corrected volumes) with predictor variables. We report type II sums of squares: the magnitude and significance of the effect of each variable in the order the variable was entered into the statistical model. In the statistical models, we first corrected for species differences by entering species as a factor. We tested for differences between queens and workers by entering caste as a factor. Finally, we tested whether species with and without morphological castes (O'Donnell 1998a; Noll et al. 2004) and independent- versus swarm-founders differed in the magnitude of queen-worker differences by using interaction terms: (caste by yes/no morphological differences) and (caste by independent/swarm-founding).

Results

Paperwasp castes differed significantly in brain architecture. Caste mean volumes for all brain structures we quantified are given in Table 1. Workers invested more than queens in visual processing brain tissues relative to antennal processing brain tissues. This caste difference was seen in both peripheral and central brain regions. In the peripheral sensory lobes, worker optic lobe/antennal lobe ratios were greater than queen ratios (Fig. 2; species differences: $F_{1,68}=12.14$, $p<0.001$; caste differences: $F_{1,68}=4.75$, $p=0.033$). The peripheral visual/antennal ratio differences were mainly due to significantly smaller optic lobes in queens (Fig. 3; $F_{1,69}=5.19$, $p=0.03$); the castes did not differ significantly in antennal lobe volume (Fig. 3; $F_{1,69}=0.425$, $p=0.52$). Polymorphic species did not differ from monomorphic species in the magnitude of peripheral visual/antennal ratio caste differences (Fig. 2; $F_{1,68}=0.17$, $p=0.69$). Independent- and swarm-founding species did not differ significantly in the magnitude of peripheral visual/antennal ratio caste differences ($F_{1,68}=2.47$, $p=0.12$), but independent-founders were among the most extremely caste-divergent species (Fig. 2).

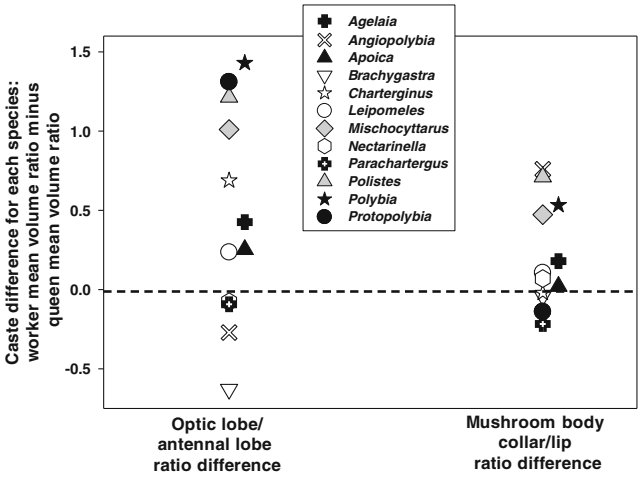

Fig. 2 Queen-worker differences in brain architecture are plotted as the difference between mean worker optic to antennal tissue volume ratios and mean queen optic to antennal tissue volume ratios. The zero line (*dashed*) indicates no mean caste difference; values above the line indicate species where worker investment in antennal tissue is relatively greater. Subject species genera are represented by symbols as shown in the boxed legend. *Gray shading* indicates independent-founding species; *unshaded symbols* indicate swarm-founding species without morphological caste differences; data points for species with morphological castes, indicated by *filled symbols*, are slightly offset for clarity

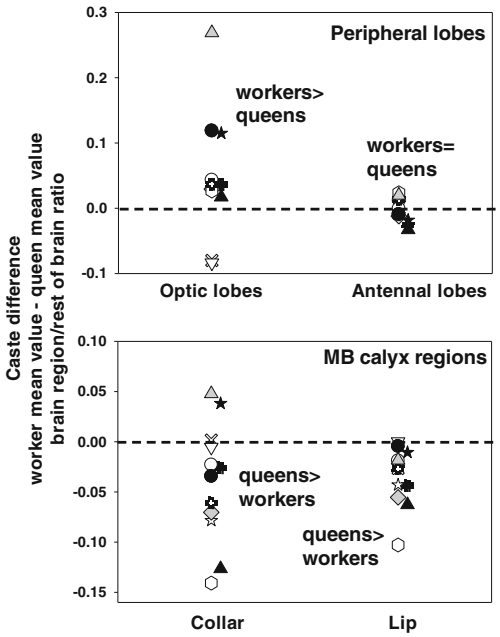

Fig. 3 Queen-worker differences in brain architecture are plotted as the difference between mean worker brain region size (corrected for brain size) and mean queen brain region size (corrected for brain size). The zero line (*dashed*) indicates no mean caste difference; values above the line indicate species where worker investment in antennal tissue is relatively greater. The patterns of caste differences supported by statistical tests are indicated next to each plot. Subject species genera are represented by symbols as shown in the boxed legend of Fig. 2. *Gray shading* indicates independent-founding species; *unshaded symbols* indicate swarm-founding species without morphological caste differences; data points for species with morphological castes, indicated by *filled symbols*, are slightly offset for clarity

Workers also had higher relative visual investment than queens in the brain's central processing regions (mushroom bodies). Worker mushroom body calyx collar (visual)/lip (antennal) volume ratios were higher than queen ratios (Fig. 2; $F_{1,68}$=7.47, p=0.008). Queens had both significantly larger mushroom body lip regions (Fig. 3; $F_{1,69}$=9.55, p=0.003) and significantly larger mushroom body collar regions (Fig. 3; $F_{1,69}$=4.19, p=0.04) than workers. The magnitude of mushroom body calyx caste differences was similar for caste dimorphic and monomorphic species (Fig. 2; $F_{1,68}$=0.078, p=0.78). Independent-founding species had stronger queen-worker differences than swarm-founders (Fig. 2; $F_{1,68}$=13.47, p<0.001).

Discussion

Caste environments

Brain architecture differed significantly between queen and worker paperwasps. Queens tended to invest less in visual relative to antennal processing tissues, both in the peripheral sensory lobes and in the central processing brain regions (mushroom body calyces). In the peripheral lobes, the queen-worker caste difference was driven mainly by lower optic lobe volumes in queens; in the mushroom body calyx, queens had both larger antennal processing lip regions and larger visual processing collar regions (O'Donnell et al. 2011).

Species and caste differences in sensory ecology are likely to be important predictors of brain investment diversity among social insects (Muscedere and Traniello 2012). Paperwasp queen and worker behavioral profiles are consistently different. Once colonies are established, the lives of queens converge on a largely nest-bound existence. Queens in mature colonies perform few tasks other than egg laying and brood care (West-Eberhard 1978; Herman et al. 2000; Noll and Zucchi 2000; Bruyndonckx et al. 2006; Chavarria-Pizarro and West-Eberhard 2010). In contrast to queens, paperwasp workers perform diverse tasks including nest defense and leaving the nest to forage (O'Donnell 1998b, 2006; Molina and O'Donnell 2008; De Souza and Prezoto 2012). Foraging and other tasks expose flying workers to elevated light levels and complex visual stimuli relative to the nest environment. Our data suggest paperwasp caste differences in behavior and sensory environment are reflected in brain anatomy, such that the cognitive requirements of each caste typically match their patterns of investment in functionally distinct brain regions.

Implications of species differences for brain development

Although we found comparative evidence for significant caste differences in sensory brain investment, species differed in the magnitude and direction of queen-worker differences. In both

peripheral and central brain regions, a third of the species we analyzed had caste differences in the opposite direction of the general pattern: queens invested more than workers in visual relative to antennal brain tissues. Some of these species differences are unexplained by our analyses. However, species differences in social structure were associated with the magnitude of caste differences. Independent-founders were among the most caste-distinct species in the peripheral lobes and had significantly stronger caste differences than swarm-founders in the mushroom body calyces.

Because we studied endpoints of adult neural development, our data cannot assess whether caste differences in brain architecture resulted from evolution or plasticity. There is evidence for both developmentally programmed, experience-expectant processes and for plastic, experience-dependent processes in adult social insect brain growth (Fahrbach et al. 1998; Farris et al. 2001). Adult queen-worker brain differences could result from caste-specific neural proliferation or caste-specific gene expression prior to adult emergence (Toth et al. 2009; Farris et al. 2011; Chen et al. 2012; Shi et al. 2013). Developmentally programmed caste differences in brain structure are seen in *Apis mellifera* honey bees (Fahrbach et al. 1998; Groh and Roessler 2008; Roat and da Cruz-Landim 2011). A non-exclusive alternative is that queen-worker differences in paperwasp brain architecture are responses to caste-specific environments that affect neuron growth (Durst et al. 1994; Fahrbach et al. 1998; Farris et al. 2001). Brain regions increase in size in response to novel cognitive challenges (Gronenberg et al. 1996; Kuhn-Buhlmann and Wehner 2006). In both bees and ants, changes in female behavior are followed by changes in brain architecture, and volume plasticity has been documented in each of the brain regions we studied (Barth et al. 1997; Jones et al. 2009; Arenas et al. 2012). Brain region volumes can also regress following reductions in cognitive demand (Gronenberg and Liebig 1999; Julian and Gronenberg 2002; Groh et al. 2006).

We suggest that if experience-expectant caste differences predominate, then caste differences would be more extreme in polymorphic species and/or in swarm- versus independent-founders. Swarm-founder colonies are larger and have stronger patterns of reproductive division of labor (Jeanne 2003). We found stronger caste differences in independent-founders. In independent-founders, a female's social environment after adult emergence, including access to nutrition and dominance interactions, plays an important role in reproductive caste differences (O'Donnell 1998b; Molina and O'Donnell 2008). The stronger caste differences in independent-founders suggest caste-divergent behavior and experience couple with adult brain plasticity to generate caste differences in brain architecture.

Acknowledgments Thanks to two anonymous reviewers for thoughtful and helpful comments on earlier versions of the paper. Emily Johnson, Joshua Matlock, Yamile Molina, Abigal Mudd, Jason Stafstrom, Eve Swearingen, and Ceri Weber assisted with histology and neuroanatomy. Specimens were collected under research permits from the Republics of Ecuador and Costa Rica and in accordance with their laws. M.R.C. was supported by an NSF Graduate Research Fellowship under Grant No. DGE-0718124; S.O'D. was supported by NSF grant IOS-1209072.

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Chapter 2. QUANTITATIVE EVIDENCE FOR POLLINATOR MEDIATED CONVERGENT EVOLUTION IN FLORAL SCENT AND MORPHOLOGY THROUGHOUT PASSIFLORA, A LARGE GENUS OF FLOWERING PLANTS

Quantitative evidence for pollinator-mediated convergent evolution in floral scent and morphology throughout *Passiflora*, a large genus of flowering plants

MR Clifford[1], JH Chau[1], N Cutlip[1], A Lance[1], N Wornian[1], Y Yuan[2], and JA Riffell[1,*]

[1]University of Washington, Department of Biology, Seattle, WA 90095 USA;

[2]University of Connecticut, Department of Ecology and Evolutionary Biology, Storrs, Connecticut 06269 USA

Running head: *Evolution of floral scent in Passiflora*

To whom correspondence should be addressed:

Jeffrey A. Riffell

jriffell@uw.edu

ABSTRACT

Pollination plays a critical role in both wild ecosystems and agricultural ones. Though past studies have included qualitative characterizations of scent as either part of broad pollination syndromes or shifts across one or a few pollinator transitions, here we demonstrated for the first time a broad, quantitative chemical component to pollination syndromes across many pollinator types and evolutionary transitions. Using integrative methods including phylogenetics, machine learning, sensory electrophysiology, and analytical chemistry, we show convergent evolution in floral morphology and floral scent, that these traits evolved in tandem to attract a given type of pollinator across *Passiflora*, a large genus of flowering plants, and that these traits have the power to predict pollinator type. We further show that such changes may be biologically relevant to available pollinators using electrophysiological methods. Though future work is required to confirm the generality of this finding in additional plant and pollinator clades, this is a critical step to better understanding the role that chemical cues play in pollinator attraction, and the evolutionary synergy they may have with morphological traits in flowering plants.

INTRODUCTION

Pollination plays a vital role in both natural and managed ecosystems (Bascompte et al. 2006; Biesmeijer et al. 2006; Brosi and Briggs 2013; Garibaldi 2014). In agricultural systems, pollinators are crucial contributors to human food security, providing ecosystem services worth an estimated $225 billion a year worldwide (Gallai et al. 2009). In wild ecosystems, pollinators are stewards of ecological diversity, mediating the reproduction of 87.5% of the ~275,000 species of extant flowering plants (Judd et al. 2008; Ollerton et al. 2011). The considerable role of pollinators in the reproductive success of modern angiosperms suggests their importance in the macroevolutionary history of these plants: the rapid radiation of flowering plants has often been attributed to their specialized associations with insect pollinators (Grant 1949; Stebbins 1970; Barrett and Willis 2001; Sprent 2005; Berendse and Scheffer 2009). On a smaller evolutionary scale, it has been demonstrated that plant fitness (Majetic et al. 2009), phenotypic selection (Parachnowitsch and Kessler 2010), and processes such as evolutionary longevity and speciation (eg. Bradshaw et al. 1995; Coyne 2004; Kay and Sargent 2009; Hermann et al. 2013; reviewed by Kay and Sargent 2009; Van der Niet and Johnson 2012a; Yuan et al. 2013a) in some flowering plants have hinged on pollinator preference, in addition to ecological and geographical factors.

Pollinators may be preferentially attracted to the flowers of particular species because of the combination of floral traits that advertise the plant's rewards (eg. pollen, nectar, oils, waxes, etc.) or the illusion thereof (Simpson and Neff 1981). The common association between functional groups of pollinators and particular suites of floral signals gave rise to the concept of pollination syndromes (Knuth 1906; Fenster et al. 2004). Though there has been some controversy over this idea (Waser et al. 1996; Ollerton and Watts 2000), a robust body of work

has investigated the relationship between floral cues, such as color and, to a lesser degree, size, shape, and texture, and a species' most effective pollinator (Harder and Johnson 2009; Kay and Sargent 2009; Yuan et al. 2013a; Campos et al. 2015). A recent meta-analysis including over 400 flowering plant species found a strong correlation between the predicted pollination syndrome and most effective pollinator, supporting the idea that convergent evolution in floral traits is driven by the preferences of the most effective pollinator (Rosas-Guerrero et al. 2014).

Though they have received less attention than visual cues, chemical cues like floral scent – the identities, relative ratios, and abundances of volatile chemical compounds emitted by a flower – play a critical role in mediating plant-pollinator interactions. Volatile chemical cues mediate, and may even be necessary to prompt, the visitation or probing behavior of many different types of pollinators (Raguso and Willis 2002; Kessler et al. 2008; Bischoff et al. 2015). Many pollinators show innate preferences for particular scents or learn to associate rewards with olfactory cues with high fidelity (eg. von Helversen et al. 2000; Guerrieri et al. 2005a; Riffell et al. 2008), and olfactory cues can improve associative learning paired with visual cues (Kunze and Gumbert 2001; Burger et al. 2010; Leonard et al. 2011; Katzenberger et al. 2013), and olfactory learning and preference have been shown to shape pollinator behavior in the field as well as in laboratory experiments (Kessler et al. 2008; Riffell et al. 2008). Furthermore, scent volatiles may be more salient than visuals as long-range cues (Bogdany and Taber 1979), particularly in environments where visual cues may be obscured (as with night-blooming or epiphytic plants), or where plants of interest are low density, patchily distributed, or bloom unpredictably in space and time (Grison-Pigé et al. 2002; Raguso 2008). And, for pollinators whose olfactory acuity outstrips their visual acuity, these chemical cues may be more physiologically and behaviorally relevant than visual cues for finding appropriate plants (eg.

Janzen 1971; Knudsen and Tollsten 1996; Sakai and Inoue 1999). Floral scent plays a role in the evolution of plant-pollinator relationships and transitions: it has been shown to be selected for by pollinators, to have an effect on plant fitness, and to be lost when pollinator associations are lost (Parachnowitsch et al. 2012a; Doubleday et al. 2013; Kuppler et al. 2016).

Although olfactory cues are demonstrably important mediators of pollination behavior for many animals, the relative difficulty of quantifying chemical cues (*versus* visual ones) has limited the field until relatively recently. In the last two decades, quantitative research on floral scent has undergone a great expansion, but has largely remained focused on the biochemistry and genetics of a few model species, particularly commercially important ones (eg. roses: Scalliet et al. 2008; Magnard et al. 2015) and has not often linked volatile emissions to pollination. In studies where pollination and evolution have been considered, research has largely focused on extremely specialized interactions (eg. Gang 2005; Mant et al. 2005; Peakall et al. 2010), a single or small number of transitions between pollinator types within a plant genus (e.g., *Mimulus:* Byers et al. 2014a; Byers et al. 2014b; *Petunia*: Klahre et al. 2011; Fenske et al. 2015; *Nicotiana*: Kessler et al. 2008; *Eucomis*: Shuttleworth and Johnson 2009; Shuttleworth and Johnson 2010; *Silene:* Waelti et al. 2008; *Linanthus*: Chess et al. 2008), or distantly-related plants that rely on a single functional group of pollinators (e.g., bats: Knudsen and Tollsten 1996; Bestmann et al. 1997; Sazima et al. 1999; von Helversen et al. 2000; Pettersson et al. 2004; moths: (Knudsen and Tollsten 1993; Levin et al. 2001; beetles: Schiestl and Dötterl 2012; hummingbirds: Knudsen et al. 2004). Studies that did investigate transitions in pollination across a broader taxonomic group characterized floral scent qualitatively rather than quantitatively (Rosas-Guerrero et al. 2014, and references therein), a method that builds human sensory bias into the conclusions. Understanding if convergent changes in floral scent occur during repeated evolutionary transitions between

pollinator types, and if these changes occur in tandem with changes in visual traits as predicted by pollination syndromes, is a key gap in the field. A critical component of this is demonstrating the role of pollinator sensation in driving these evolutionary processes in plants.

The passionflowers (*Passiflora)*, a primarily tropical genus of flowering plants with over 500 species and observed pollination by several animal functional groups (Fig. 1)(Krosnick et al. 2013; Abrahamczyk et al. 2014), provide an excellent opportunity to explore how floral scent and other cues change during evolutionary transitions between pollinator types. Furthermore, the recent availability of a diverse array of chemical, electrophysiological, and computational techniques enables us to build an understanding of floral trait evolution from both the perspective of the plant and, critically, from the perspective of the pollinators driving trait changes, a gap that is infrequently bridged (but see Stökl et al. 2010; Byers et al. 2014b; Friberg et al. 2014). In this study, we integrate phylogenetics, comparative biology, sensory neurobiology, analytical chemistry, and machine learning techniques to quantitatively test for a scent component to pollination syndromes. Specifically, we ask whether the same quantitative patterns in floral scent chemistry recapitulate themselves during independent transitions from one pollinator to another, and whether such convergent scent evolution occurs in tandem with convergent morphological evolution. The integration of chemical and morphological cues to attract pollinators would suggest a critical and largely overlooked scent component to our traditional, visual understanding of pollination syndromes. We also assessed the biological relevance of observed scent cues to pollinator associations by testing the correlation of physiological responses to these cues with observed pollination behavior within and between groups of insect pollinators, as more physiologically salient cues have been shown in past studies to have a greater effect on

pollination behavior and to be learned more quickly or with stronger association (Guerrieri et al. 2005b; Daly et al. 2007; Katzenberger et al. 2013).

MATERIALS AND METHODS

For additional details on *Plant Rearing, Voucher Preparation, Volatile Collections and Analysis*, and *Phylogenetic Data*, please see Materials and Methods section in Supplemental Information.

Floral Specimens

69 species of *Passiflora* were grown from cuttings, seedlings, or seed procured from university collections, personal collections, seed banks, and commercial growers in a controlled greenhouse at the University of Washington. For a table of voucher accessions and corresponding Genbank accession numbers, see SI Table 1.

Morphological Characters

Color, shape, size, pattern, and sex organ orientation (see SI Table 2) were measured from flowers using a millimeter ruler if continuous or scored by eye if discrete. To increase overlap between the morphological data set with the phylogenetic data set, our morphological data was combined with a larger data set available from the literature (Christensen 1998; Varassin et al. 2001; Ulmer 2004; Jorgensen et al. 2012).

Volatile Headspace (Scent) Collection and Analysis

Scent was collected from greenhouse-grown *Passiflora* using a push-pull system (*sensu* Raguso and Pellmyr 1998; Riffell et al. 2008; Byers et al. 2014b) for 24 hours, and then eluted into

HPLC-grade hexane (Sigma-Aldrich, St. Louis, MO USA), and run on a gas chromatograph-mass spectrometer (GCMS, 7890A GC paired with 5975C MS; Agilent Technologies, Palo Alto, CA, USA) to determine the abundance, identity, and relative ratios compounds making up each scent sample (please see Supplementary Information for details).

For each scent sample, GCMS peaks were quantified and manually assessed using Agilent Chemstation software (Agilent Technologies). A custom software in Python and R (available upon request) was used combine chemical synonyms in the mass spectrum peak matches using data from the National Institute of Standards (NIST) Chemical WebBook (Linstrom and Mallard 2015), since results for the same chemical were returned by Chemstation/NIST database with many synonymous names; to choose the most likely identity for each chromatogram peak based on matching between the mass spectrum results and the Kovats Index of the GC retention time ((Arn and Acree 1998; Adams 2001; Skogerson et al. 2011) and standards run in the lab); to remove chemical contaminants; and to combine all data into a single chemical matrix.

Genetic Data and Phylogeny

Sequence data from two plastid loci (*trnT-trnF*, *ndhF*) and two nuclear loci (ITS, *ncpGS*) were used to infer a phylogeny for *Passiflora*. Sequences were gathered from Genbank or sequenced using tissue from greenhouse plants or herbarium specimens.

For each locus, sequences were aligned using MAFFT ver. 7 (Katoh and Standley 2013). The default strategy and parameters were used for *ndhF* and *ncpGS*, and the E-INS-i strategy was used for *trnT-trnF* and ITS. Alignments were inspected and manually edited in Se-Al v2.0a11. Bayesian phylogenetic analyses were performed using MrBayes 3.2.3 (Ronquist et al.

2012) on CIPRES Science Gateway with a concatenated sequence dataset and the GTR+G model of nucleotide substitution. Model parameters were unlinked across loci. Analyses were run for 30,000,000 generations with sampling every 3,000 generations. Convergence was assessed by checking the average standard deviation of split frequencies in MrBayes, and the estimated sample sizes of parameters and trace of log-likelihood values in Tracer v1.5 (Rambaut and Drummond 2009). A burn-in consisting of the initial 25% of trees was discarded before constructing the majority-rule consensus tree (please see Supplementary Information for details).

Modeling, Analyses, and Sampling Scheme

The multiple objectives and multiple types of data collected for this study required multiple types of statistical, clustering, and machine learning methods to analyze, as different methods had different distributional and other assumptions. As a first step to determine whether differences in floral traits (morphology and scent) are explained by pollinator type, we used dimensionality reduction/clustering methods and permutational statistical methods to visualize patterns in multivariate data, discern if variables like relatedness or pollinator explained observed patterns, and determine if clusters were significantly different from one another (Morphological data: multivariate normal continuous + discrete characters, clustering using hierarchical clustering with Gower's distance, visualization with PCO, PERMANOVA significance testing. Scent data: sparse, zero-inflated raw multivariate data, clustering with Bray-Curtis dissimilarity for percentage and raw or Jaccard's for binary, visualization with NMDS, distribution-free ANOSIM significance testing). Because none of our data fully conformed to multivariate normality, we did not use PCA for clustering or to determine which variables were key contributors.

We next used the machine learning algorithm random forest to elucidate which traits contributed most to discrimination between flowers pollinated by different functional groups, and to see how well floral morphological and scent trait data could actually predict pollinator. The machine learning algorithm random forest uses an ensemble of decision trees, each created from a bootstrap sample the input data (two-thirds of input data in our imbalanced sampling scheme; 4 bat-pollinated, 4 wasp-pollinated, 8 bee-pollinated, 8 bird-pollinated for our balanced sampling scheme), to classify data into categories based on the vote of the ensemble; it does not have distributional requirements, does not typically over-fit, and can create an estimate of its own accuracy as it is run – called out-of-bag error – by assessing in what proportion of trees' data points that were outside of the bootstrap sample for that set of trees were classified incorrectly (Breiman 2001). Mantel tests were used to detect phylogenetic signal in multivariate data sets and test for correlation between multivariate data sets while controlling for phylogenetic contribution, each run with 999 permutations.

Maximum likelihood methods with an equal rates model were used for ancestral state reconstruction (Yang et al. 1995). For details on R and Python packages used to clean, gather, and analyze data, see "Programming for Data Gathering, Data Cleaning, and Statistical Analysis" in Supplementary Information Materials and Methods. Our final ancestral state reconstruction used maximum likelihood methods on species for which we had sequence data and had either pollinator observations or could infer pollinator using floral morphology (see results for details on the high fidelity of this prediction using random forest with morphological data). Inferring pollinator transitions and ancestral state using only pollinator observations in the literature may be problematic. The pollination data set includes a relatively small number of species compared to the whole genus, and ancestral state reconstruction can be sensitive to

missing species (*sensu* Blumstein et al. 2015). Error may also be introduced into the observed pollinator data set because visitation data is included along with more robust studies of pollinator effectiveness (eg. contact of pollinator with sex organs, pollen load deposited; see SI Table 3) because these robust studies are comparatively rare; recorded visitors are not necessarily pollinators or may not be the most effective pollinators for that particular flower species. To get an ancestral state reconstruction with increased representation of older subgenera and fewer species with missing data without using techniques that assume no trait information (eg. assigning equal prior probabilities to each state for species without pollination observations; as in implementation in Revell, 2012), we used random forest to predict the pollinator of all species for which we had both morphological and phylogenetic data (132 species). Using inferred pollinator increased coverage from under 10% to almost a quarter of known species in the genus. A caveat is that certain groups are sampled in greater depth (eg. supersection *Tacsonia*, (Abrahamczyk et al. 2014)), while others (eg. subgenus *Tetrapathea*) were still not sampled because of their relative rarity.

For additional details on sampling and which species were included in each analysis, please see SI Materials and Methods and SI Table 5. For clarity, the majority of the results from the statistical tests are placed in a single document in SI (**SI statistical tables document**).

Model Pollinator Electrophysiological Responses to Floral Scents and Individual Chemicals

To determine whether model pollinators (bumblebee [*Bombus impatiens*], carpenter bee [*Xylocopa californica arizonensis*], moth [*Manduca sexta*]) and a *Passiflora*-associate (butterfly [*Heliconius melpomene*]) differentially responded to their cognate floral scents, and identify if these insects exhibit an olfactory bias for individual volatiles in the scent that might be typical of

a chemical syndrome, electroantennograms (EAGs) and gas chromatography coupled to electroantennogram detection (GC-EADs) experiments were performed. EAGs were performed by connecting electrodes to the insect antennae and recording antennal responses to the different floral scents; responses are thought to reflect the summation of all olfactory receptor responses on the insect antenna. Similarly, GC-EADs were performed by recording antennal responses, but here antennal responses were to the isolated volatiles from the floral extracts that elute from the gas chromatograph; this method allows identification of the chemicals in a complex floral scent that the insects respond to. For full details on *Insect Rearing*, *Olfactory stimulation* and *Gas Chromatography linked Electroantennogram detection (GC-EADs)* and *Electroanntennography (EAGs)*, please see Supplemental Information.

RESULTS

MORPHOLOGY

Modeling Morphological Traits With and Without Pollination Information (Unsupervised Clustering and Supervised Learning)

As a first step, we used hierarchical clustering and PERMANOVA statistical testing methods to examine similarities and differences in the floral morphology of *Passiflora* species that are pollinated by distinct types of pollinators. In almost all cases, *Passiflora* that were pollinated by different pollinator types were significantly different from one another (Fig. 2)(PERMANOVA, **SI statistical tables document** (abbreviated to "SI doc" and table number going forward) **t1**; sampling, **SI doc t2**). Exceptions were between bee- and moth-pollinated plants and between bird-pollinated plants and those pollinated by multiple pollinators (birds, butterflies and bees, or BBB). Multiple evolutionary transitions from the ancestral state of bee-pollination ((Janzen

1968); see Morphology, Pollination, and Phylogeny section) to each pollination syndrome (bird, bat, moth, wasp, multiple) were included in this analysis. Mantel tests confirmed a significant correlation between floral morphology and observed pollinator both with and without taking phylogenetic relatedness into account (Figs. 1,2; **SI doc t3**).

We next used the random forest algorithm on the 64 *Passiflora* species for which we had pollinator observation data (see SI Table 3) and morphological data (see SI Table 2) to understand how well floral morphology could predict pollinator and which morphological traits were most important for predicting pollinator.

The morphological random forest with raw data (pollination groups represented as they were in our sample, n = 64 species; variables tried per node = 3, number of decision trees = 2000) had an accuracy of approximately 80% (out-of-bag (OOB) error rate of 20.31%; **SI doc t4**) for classifying flowers into the correct (of five) pollination category. Because the orientation of sex organs is mechanically important in pollination, but may not be an important visual cue to pollinators, we also ran the model excluding sex organ orientation. Without sex organs included as a factor, the model had an accuracy of approximately 71% (OOB error rate of 29.69%; **SI doc t5**). Likely because they made up most of the training data, bee and bird pollination were most accurately predicted; moth-pollination could not be predicted because of the small number of species representing this group. The errors in classification made in the model reflected the proximity of clusters found in the unsupervised learning analyses: bee was most commonly mistaken for wasp and vice versa, while bat was mistaken for bee or bird depending on the species. Similar patterns in classification errors (confusion) held when sex organ was removed as a factor from the model, except that bee-pollinated flowers, with their relatively diverse morphology, were mistaken for more pollinator groups when sex organ was excluded. We ran

the same analysis including only the species that appear in all data sets (pollinator observation, floral scent chemistry, floral morphology, phylogenetic relatedness; n = 35) to ensure that accuracy of the models was not due to the larger size of the morphological data set relative to the scent one; we obtained a similar model with similar error patterns (**SI doc t6**). Repeating these analyses excluding moth species (n = 62) and increasing the number of variables tried per node to 10 did not affect overall OOB error, class error rates, or confusion, and produced identical pollinator predictions for species without observed pollinators.

We next constructed morphological random forest models using more balanced samples from each pollination group for each run to see if this would decrease error rates for less common pollinator groups (6 wasp-, 6 bat-, 8 bee-, and 8 bird-pollinated species were randomly selected for tree run; n = 62 species, variables tried per node = 10, number of decision trees = 2000). Moth-pollinated species were excluded because our sample was too small to facilitate effective down-sampling of other groups. Using the full data set with balanced sampling lowered overall error rates and considerably lowered error for bat- and wasp-pollinated species, predicting pollinator with over 85% fidelity (OOB error = 14.52%, **SI doc t7**). Removing sex organ from the predictors increased overall OOB error by about 10%, and particularly increased error rates for bat- and bird-pollinated species (OOB error = 22.58%, **SI doc t7**). Repeating this analysis with only the species that appear in all data sets yielded comparable results to the full analysis (n = 35; OOB error = 14.29%, **SI doc t8**).

Relative Importance of Morphological Traits in Predicting Pollinator

Which morphological features of the flowers best predicted these pollinator relationships? In the balanced random forest, sex organ orientation, followed by the main color of the petals and sepal

and petal length (a proxy for flower size) were the most important factors for predicting pollinator using both common random forest importance metrics (mean decrease in accuracy and mean decrease in that predictor [GINI ratio]). These were followed by metrics patterning and orientation of the corona, a ring of appendages extending from the corolla that is typical of the *Passiflora*. Less important for classification were metrics for the less prominent colors on the flower (corona color, the presence of red stems or bracts), shape (ratio of flower diameter to petal length, degree of sepal reflexion, and length of petal tube), and the number of coronal layers. Cross validating the random forest model showed that using sex organ orientation alone had a classification error only 10% worse than a model using between 2 and all 14 traits; using both sex organ and color was only 1.6% less accurate than using all 14 traits (**SI doc t9**). Again, because sex organ orientation seems unlikely to act as a major cue for pollinators, we repeated this analysis without including sex organ orientation. Main color alone could classify with 50% error, and after including the top 6 metrics (color, pattern, and size metrics) this error dropped to approximately 27%, or just 5% worse than the full model (**SI doc t10**).

POLLINATION TRANSITIONS ACROSS THE PHYLOGENY

To understand how much of floral scent evolution is due to selection by pollinators, it is important to elucidate where and in what direction pollination transitions occur in the *Passiflora* phylogeny (comprising five distinct subgenera with over 560 species (Krosnick et al. 2013)) and ensure that many of these transitions are captured in our trait data. We also set out to verify that the ancestral pollination state in this genus is bee-pollination (Janzen 1968), as this may be most common in plant systems (Bawa 1990) and additional or atypical constraints on floral trait evolution may exist in clades where the ancestral state is pollination by a more derived pollinator

(eg. *Ferraria*: Goldblatt et al. 2009).

Pollinator observations from the literature include data on 76 species, primarily from the largest subgenera, *Passiflora* and *Decaloba*, but also from two closely related species in the small subgenus *Deidamioides* (SI Table 3). Our phylogeny, which sampled over 240 species (SI Fig. 1), included 58 species for which there were pollination observations: with substantial representation of those whose primary pollinator was a bee or bird, and smaller numbers of species whose primary pollinator was bat, wasp, or moth + bee or wasp (SI Table 3). In this analysis, the ancestral state for *Passiflora* was most likely bee, but bat is a close second (Fig. 1). Given the relative rarity of bat pollination in the genus, this is likely an artifact of bat-pollination observed in *Deidamioides* and the lack of observations from *Astrophaea*, which branches from one of the deepest nodes in the tree and is sister to the clade containing all other subgenera. In this analysis, which had many missing species, transitions from bee to bird and bee to wasp were most common, followed bird to bat, and bee to bat and bee to moth (Fig. 1). Also yielded by the model were transitions that are unlikely to have occurred and are likely an artifact of sampling error in our data set, such as moth to bird and bird to wasp.

To get an ancestral state reconstruction with increased representation of older subgenera and fewer species with missing data, we used random forest to predict the pollinator of all species for which we had both morphological and phylogenetic data (n = 132 species). To be conservative, we used predicted pollinators from the model using raw data rather than balanced samples, as the predictions from this model and the balanced model agreed 93% of the time, but the model using raw data proposed 2 fewer transitions from bee pollination than did the balanced model in species with phylogenetic data. In this larger data set, there were 66 bee, 45 bird, eight

bat, 12 wasp, and one moth -pollinated species and a total of 13 bee to bird transitions, 6 bee to wasp, 3-4 bird to bat, 1-2 bee to bat, 1 bee to moth, and 1 wasp back to bee (SI Fig. 2).

CHEMISTRY

Unsupervised Scent Clustering

As a first step to determine whether *Passiflora* floral scent significantly clustered according to pollinator type without using pollinator as an explicit classifier, scent data was analyzed several different ways, by: (1) scent composition (raw data and percentage), (2) presence/absence of chemicals; (3) number of identified chemicals in the scent; and (4) total scent emissions. In all analyses, there were significant differences in scent by inferred pollinator type (Fig. 3; **SI doc t11-16, and 19**). Statistical tests using the scent composition data for both those flowers with observed pollinators (n = 38), and those inferred by the change-conservative floral morphology random forest to increase the size of our individual chemical abundance - pollinator data set (n = 70), showed significant scent differences between pollinator groups (**SI doc t14, 15)**. Analyzing the data (SI Table 4) according to presence/absence and percentage abundance of chemicals in the scent – thus ensuring differences were not driven by the differences in emission, but also by the chemical composition of the scents – produced similar results (**SI doc t13, 14**). Mantel tests confirmed a significant correlation between floral scent composition and inferred pollinator both with and without explicitly taking phylogenetic relatedness into account (SI Fig. 3)(n = 61; Mantel chemical abundances ~ pollinator inferred by morphology: R = 0.097, p = 0.019; Mantel chemical abundances ~ phylogeny + pollinator inferred by morphology: R = 0.097, p = 0.016).

Absolute floral scent emission rate was significantly explained by inferred pollinator type, with bee and bat flowers emitting approximately 10 times more than bird and wasp flowers

on average (Fig. 3D)(Type III ANOVA for unbalanced samples and adjusted Tukey-Kramer post hoc tests for unbalanced samples, $p < 0.0001$; $n = 70$; **SI doc t15 and t16**). Repeating the analysis with phylogenetic correction yielded similar results: inferred pollinator still significantly explained daily scent emission rate after taking relatedness into account (phylogenetic ANOVA, $p = 0.045$, $n = 65$). However, post-hoc pairwise comparisons yielded slightly different results from analyses that did not apply a phylogenetic correction: bat-pollinated flowers tended to emit more than their ancestors and bird-pollinated flowers evolved to emit significantly less, but the evolution of wasp pollination did not lead to significant changes in scent emission rate (phylogenetic generalized least squares (PGLS) $\log_{10}$(24-hour emission) ~ pollinator: **SI doc t17**; pairwise contrasts with phylogenetic ANOVA: **SI doc t18**). The moth-pollinated group was excluded because it consisted of one data point.

The absolute number of chemicals making up a given floral scent was also was significantly explained by inferred pollinator type (Type III ANOVA for unbalanced samples, $p = 0.002$; $n = 70$; **SI doc t19**), but most pairwise comparisons were not significant (Tukey-Kramer post hoc tests for unbalanced samples; $n = 70$; **SI doc t20**). Bee-pollinated flowers emitted significantly more chemicals than wasp- or bird-pollinated flowers, but the bat-pollinated flowers were not significantly different from either group. However, repeating the analysis with phylogenetic correction showed a non-significant overall contribution of pollinator to the number of chemicals in a species floral scent (phylogenetic ANOVA $p = 0.178$, $n = 65$; PGLS estimates of mean number of chemicals emitted by inferred pollinator: **SI doc t21**). Only a single post-hoc test showed a significant difference in the number of chemicals emitted: bird-pollinated flowers evolved to emit fewer chemicals than bee-pollinated ones (**SI doc t22**).

<u>Modeling Scent Including Pollination Information</u>

As with the morphological data, we used random forest to whether scent data could predict the type of pollinator. We used species for which we had pollinator observations (n = 38) as a training set and species for which we had scent data as a test set (n = 72). Observed pollinator was predicted correctly with about 65% fidelity overall (OOB error rates 35-39%, 20,000 decision trees, 500 variables tried at each split), but only bee- and bird-pollinated flowers were reliably discriminated from one another using the scent chemical composition, percentage, and presence/absence scent data and for both raw and balanced sampling (random forest bee *vs*. bird with a variety of parameters, sampling schemes, and scent data sets: **SI doc t23**; scent composition data ~ inferred pollinator random forest: **SI doc t24**; presence/absence scent data ~ inferred pollinator random forest: **SI doc t25**; percentage scent compositon ~ inferred pollinator random forest: **SI doc t26**). When we increased the size of our training set using the pollinator inferred by the morphological random forest, the chemical random forest performed similarly. When we used fewer trees and fewer variables tried per split (2000 and 22 respectively), OOB and class error rates were almost identical for the larger inferred pollinator data set, but much higher for the smaller observed pollinator data set. Aggregating chemicals by broad (eg. terpene, oxygenated aromatic) or specific chemical class (eg. lactone, alkane ester) increased error rates in all cases, suggesting the importance of individual chemical identity over chemical class. Random forest analyses also quantified which chemicals were most important in discriminating flower types, with the caveat that our model was biased towards discriminating between bird- and bee-pollinated flowers since they were most common in our data set. β-Ocimene was consistently the most important odorant for discrimination, in addition to other terpenes, oxygenated terpenes, and oxygenated aromatics and a handful of oxygenated alkanes.

Chemicals Unique to a Pollinator Type

Because the data sets for pairwise comparisons besides bee-bird were very small and random forest performs poorly on very small data sets (Verikas et al. 2011), we investigated how the scents of *Passiflora* species pollinated by other pollinators were chemically distinct by looking at which chemicals were unique to them. Of the 508 chemicals in our data set (SI Table 4), 214 chemicals were unique to bee-pollinated species, 32 to bat-pollinated species, 17 to wasp-pollinated species, and 14 to bird-pollinated species (using pollinator inferred by floral morphology). However, when we normalize for the different numbers of species in each group, bat-pollinated species contributed the largest number of unique chemicals/species at 6.4, followed by bee at 5.14, then wasp at 1.7. Bird-pollinated species only contributed 0.875 new compounds per species. Because the median number of chemicals emitted by bee- > bat- > bird- > wasp-pollinated species and the mean number of chemicals emitted by bee and bat, and by bat, bird, and wasp are not significantly different from one another, this potential difference in unique chemicals emitted is unlikely to be explained by the number of chemicals emitted.

Notable chemicals that were unique to bat-pollinated species included several lactones, an oxime, and oxygenated alkanes. Most had odors described as "fermented," "fatty" or like chicken fat, "creamy", or "fruity" to the human nose (Goodrich et al. 2006; Company 2015). The terpenes that were unique to bat-pollinated species were all described as "woody," (Company 2015) in contrast to the "sweet" or "fresh" terpenes that were more common in bee flowers (Company 2015; as in Harborne 2001; Goodrich and Raguso 2009) (chemical unique to bat-pollinated *Passiflora*: **SI doc t27**).

The set of 17 chemicals unique to wasp-pollinated species was dominated by alkanes on which there was no organoleptic information available (2-Ethyl-4,6-dimethyltetrahydropyran; 5-tridecene; 6,9-heptadecadiene; bicyclo[2.2.2]octane, 2-methyl; bicyclo[5.3.0] decane; decane,

2,3,8-trimethyl; 3,4-octadiene, 7-methyl), as well as a lactone, two fruity-smelling aldehydes (Company 2015), and a strong fecal indole relative (Schiestl and Dötterl 2012) (**SI doc t28**).

INSECT ELECTROPHYSIOLOGICAL RESPONSES TO FLORAL SCENTS

To determine whether the olfactory systems of model pollinator species (bumblebee, carpenter bee, moth, and butterfly) could detect differences between floral scents, and to establish whether a given pollinator type has evolved to detect certain key volatiles that are typical of that pollinator syndrome, we conducted electroantennograms (EAGs) and gas chromatography coupled with electroantennogram detection (GC-EAD). EAGs were conducted using model species of bees (*B. impatiens, X. californica arizonensis*) and moths (*M. sexta*), as well as pestiferous butterflies (*H. melpomene*; a *Passiflora*-associate), to elucidate if these insects exhibited larger antennal responses to flower species pollinated by their own functional group (sample of scents from plant species pollinated by: bat (2 sp.), bee (17 sp.), bird (8 sp.), wasp (3 sp.), and moth (1 sp.)). Differences in the magnitude of responses between individual insects were still large, so we used generalized linear mixed effects models (GLMMs) to account for these random effects before looking for differences based on the pollinator of the presented scent. For both bumblebees and moths, the pollinator of the plant which supplied the odor better explained the antennal responses than individual variation alone (ANOVA to compare null model, $p < 0.0001$ for all insect species, $n_{M.\ sexta} = 8$, $n_{B.\ impatiens} = 9$, $n_{H.\ melpomene} = 5$). *Bombus impatiens* responded significantly more to all types of floral odors except wasp-pollinated scents than to the negative controls (hexane and mineral oil), but only responded significantly more to flowers from to bee-pollinated species than to vegetative scents (Fig. 4; **SI doc t29**). Responses to bat, moth, and bird flowers appeared intermediate between bee flowers and vegetative scents,

but were not significantly different from either (**SI doc t29**). By contrast, *M. sexta* responses did not respond to any type of floral scent significantly more than to vegetative scents (Fig. 4; **SI doc t30**). We were only able to include a single moth-pollinated species, and did not have the power to test for significantly higher responses by *M. sexta* to moth-pollinated flowers. However, that response to the moth-pollinated species by *M. sexta* was high compared to that of *B. impatiens*, where response to the single moth-pollinated species was comparable to their responses to scent-poor bird-pollinated species, suggesting this larger response by *M. sexta* is due to the composition of the floral scent, rather than scent intensity, which should affect responses by both types of pollinators equally. *Heliconius melpomene*, which does not visit *Passiflora* flowers but uses *Passiflora* spp. as host plants, showed larger responses to vegetative odors relative to the negative control than *B. impatiens*, but no category of responses were significantly different from one another (*Heliconius*-*Passiflora* GLMM; **SI doc t31**).

To determine whether the model pollinators differentially respond to individual chemicals that are found in flowers pollinated by the different pollinator types, and to establish whether those bioactive volatiles could also be used to predict the pollinator type for the different *Passiflora* scents, we presented the insects with a diverse panel of individual odorants – both synthetic and natural volatiles from the GC. Results from these experiments demonstrated that moths and bees responded significantly different from one another (**SI doc t32-t35**; SI Figs. 4,5). By contrast, results from the GC-EAD experiments showed carpenter and bumble bees responded to the same compounds within the *Passiflora quadrangularis* floral scent; in particular, oxygenated terpenes and oxygenated aromatic compounds (**SI doc t33**). We next repeated the individual chemical multivariate analyses integrating our results from bee GC-EADs and single odorant EAGs to identify whether those volatiles could also predict between

pollinator types. When we filtered the chemicals included in the multivariate analysis to include only chemicals that were bioactive, there was a significant correlation in predicting bee-pollinated flowers (bee filtered chemical (EAG and GC-EAD) data ~ inferred pollinator; ANOSIM; overall: $R > 0.420$, $p = 0.001$; **SI doc t34, t35**).

CHEMISTRY AND MORPHOLOGY TOGETHER

The Importance of Morphological and Chemical Floral Traits in Predicting Pollinator

Given differences in floral scents, and the bioactivity of the scents for the different model pollinators, how then might floral scent chemistry and floral morphology together predict the pollinator type? In random forest models using the same *Passiflora* species (n = 58) with pollinator observations (n = 36 total species; 4 bat sp., 14 bee sp., 14 bird sp., 3 wasp sp., 1 moth sp.) as the test and training sets respectively, multivariate morphology predicted pollinator with lower error than scent data. In random forest models where chemical and morphological data sets were combined (2000 trees, 22 variables tried at each split), every morphological trait was more important in classification than every individual chemical trait, the OOB error was comparable to models using morphology alone (~25%) and cross validation showed that adding the chemical data to the morphological data did not improve the error rate (**SI doc t36**). Removing the single moth species from the analysis to enable down-sampling, as well as using a balanced sample, more trees, and more variables tried per split (4 bat, wasp and 8 bee, bird sampled/tree, 2000 trees, 500 variables tried per split; total test set n = 35) improved overall error to 17-20%, and decreased class error for bee and wasp flowers by 33 - 67%; and, though morphological traits like petal/sepal color, sex organ orientation, and coronal pattern dominated chemical traits in importance, some chemical traits were more important for classification than some morphological traits.

Correlation between Morphological and Chemical Traits and the Contribution of Phylogeny

Using the 57 species that overlapped between our chemical, morphological, and phylogenetic data sets, which represented many pollination transitions across the *Passiflora* phylogeny, we performed Mantel and partial Mantel tests to see if multivariate chemistry and multivariate morphology are correlated. Floral morphology and scent chemistry were significantly correlated, but both exhibited significant phylogenetic signal (overlapping chemical, morphological, and phylogenetic data set, n = 57; **SI doc t37**). To ensure that the correlation between morphology and chemistry was not due to shared phylogenetic signal, we performed a partial Mantel test taking the contribution of phylogenetic relationships into account; results from this analysis revealed that morphology and chemistry were still correlated with 10% explanatory value (n = 57, Mantel R = 0.101, p = 0.008, **SI doc t37**). The highly overlapping confidence intervals between the test taking phylogeny into account and the test not taking it into account suggests that the observed correlation between morphology and chemistry exists largely independently of phylogeny. We performed a similar test taking pollinator into account before correlating floral chemistry and morphology. This showed that the correlation between morphology and chemistry is no longer strong or significant after taking inferred pollinator into account (n = 57, Mantel R = 0.032, p = 0.24; **SI doc t37**), though we caveat this because pollinator for this test was inferred by morphology.

DISCUSSION

Based on quantified floral traits from phylogenetically diverse *Passiflora* species, correlation between those traits and most effective pollinator, and pollinator physiological responses to those traits, we found evidence for pollinator-mediated convergent evolution in both floral morphology

and floral scent, suggesting the existence of a quantitative chemical aspect to pollination syndromes. To our knowledge, this is the first study to demonstrate a quantitative chemical aspect to pollination syndromes across many evolutionary transitions between multiple types of pollinators, and the first to use an integrative approach that brings together floral chemistry, floral morphology and pollinator olfaction (or chemoreception).

Convergent Evolution in Floral Morphology

Mirroring patterns found across other plant systems, *Passiflora* species throughout the phylogeny showed convergence in floral morphology that was well-explained by their observed pollinator type, confirmed that floral morphology could predict most effective pollinator, and suggested a role for both ethological and mechanical isolation in the evolution of convergence (Grant 1949; Fenster et al. 2004; Rosas-Guerrero et al. 2014).

As inputs to the morphological clustering, we used traits representing floral color, size, shape, orientation, and pattern, each of which has been shown to individually influence pollinator choice in other plant systems (color: Bradshaw and Schemske 2003; Streisfeld and Kohn 2005; Hoballah et al. 2007; Dell'Olivo and Kuhlemeier 2013; shape: Fulton and Hodges 1999; Alexandersson and Johnson 2002; Strakosh and Ferguson 2005; Whittall and Hodges 2007; Owen and Bradshaw 2011; size: Thomson 1988; Conner and Rush 1996; Grindeland et al. 2005; orientation: Fulton and Hodges 1999; Hodges et al. 2002; pattern: Goyret 2010; Owen and Bradshaw 2011; Shang et al. 2011; Yuan et al. 2013b); both closely and distantly related species pollinated by the same guild clustered together in groups statistically distinct from one another. Correlation between morphological traits and observed pollinator remained with and without taking phylogenetic relatedness into account, consistent with convergent evolution for attracting

a given pollinator type. The positions of the morphological clusters relative to one another comported to our expectations: Bird and bee were the largest and most divergent clusters. BBB (species with multiple pollinators, combinations of bee, bird, and butterfly) and bat were intermediate between the bird and bee clusters in morphospace. Wasp-pollinated plants clustered morphologically within the bee-pollinated plants, but in a distinct subspace; the shape of the flower did not need to change much to accommodate a pollinator of similar shape, but sensory cues like color and size differed to attract the wasps.

Historically a major challenge to pollination syndromes has been that, while correlations between floral morphology and visitors may exist in some cases, floral visitors could not be consistently predicted from floral traits (eg. Waser et al. 1996; Ollerton et al. 2009; reviewed by Rosas-Guerrero et al. 2014). However, recent meta-analyses including over 400 plant species demonstrated that, while the exact species of the most effective pollinator may not be predictable, its functional group could be dependably predicted from suites of floral traits, and that secondary visitors were often ancestral pollinators rather than random generalists (Rosas-Guerrero et al. 2014). Furthermore, experimental evolution studies have provided evidence that pollinator choice tends to move floral phenotypes back towards the value predicted by their syndrome (Campos et al. 2015), and that selective pressure can shape phenotypic convergence in flowers from wildly distinct pathways and starting points (Ng and Smith 2016; Strelin et al. 2016). In predictive models with *Passiflora*, floral morphology could predict pollinator type with 85% fidelity, or with 78% fidelity when only traits likely to be used as cues by pollinator, such as color, size, and shape, were included; of the traits likely to be used in pollinator choice, floral color was the most powerful predictor, followed by pattern and size. The increase in predictability when including traits likely to aid in pollen placement rather than pollinator

preference may suggest a role for both mechanical and ethological isolation in this system (Grant 1949; Culbert and Forrest 2016), though the contribution of traits likely influencing pollinator choice is much larger (Ramsey et al. 2003; Xu et al. 2011). These results add support to the well-established concept of the morphological pollination syndrome, demonstrating that similar patterns in floral morphology evolved convergently to attract a particular pollinator type. Furthermore, they confirm the still somewhat controversial idea that floral morphology can be used to predict the most effective pollinator for a given angiosperm species.

Many Pollinator Transitions in the Study Genus, *Passiflora*

Ancestral state reconstruction using observed pollinator confirmed that there were many evolutionary transitions from one type of pollinator to another in the *Passiflora*. Evolutionary transitions found in the *Passiflora* included bee to bird, bee to bat, bird to bat, bee to wasp, and perhaps bee to moth, echoing the common transitions found across many other taxa (Grant 1949; Van der Niet and Johnson 2012b; Rosas-Guerrero et al. 2014). Furthermore, these analyses verified that the ancestral state of this genus is bee-pollination (as asserted by Janzen 1968), which is likely to be most common in plant systems (Bawa 1990) and additional or atypical constraints on floral trait evolution may exist in clades where the ancestral state is pollination by a more derived pollinator (eg. *Ferraria*, (Goldblatt et al. 2009)). Together, this confirmed that *Passiflora* is an ideal system to study typical patterns of change and convergence in floral traits across many different pollination transitions. The ancestral state reconstruction was repeated using the low-error morphology model to predict pollinator for additional species in order to increase sample size for chemistry-pollination analyses. The ancestral state reconstructions with observed and inferred pollinator showed similar results and confirmed that many evolutionary

transitions from one pollinator type to another were represented in our chemical and morphological trait data.

Evidence for the Importance of Floral Scent in Pollination Syndromes

Our chemical, morphological, phylogenetic, and electrophysiological results provide quantitative evidence for a volatile chemical aspect to pollination syndromes for bee-, bird-, bat-, and wasp-pollinated flowers from the perspective of both plant and pollinator. All three of our predictions for the existence of a scent aspect to pollination syndromes were satisfied: (1) floral scent chemistry clustered based on pollinator type across multiple evolutionary transitions, suggesting convergent evolution, and that floral scent chemistry may predict pollinator type; (2) differences in floral scent were physiologically relevant to pollinating insects in ways material to pollinator-mediated speciation; and (3) species' floral morphology and floral scent chemistry were correlated with one another, suggesting that these traits evolved together to attract a given pollinator type.

Clustering and predictability suggested convergent evolution of floral scent by pollinator type

Echoing the findings in floral morphology, floral scent chemistry clustered by pollinator in all three aspects of floral scent: abundance, relative ratio of compounds, and compound identity (presence/absence). Pollinator significantly explained chemical clustering overall, and pairwise comparisons showed that scent clusters were chemically distinct from one another with few exceptions (wasp-bat comparisons, though plant and pollinator phenology – with bat flowers open at night and wasp flowers open in the day – may make this moot; moth could not be tested because we had samples from only one species visited by moths ((Ulmer 2004), see SI Table 3).

Other results validate and extend patterns others have found at a gross level to a larger quantity of transitions and pollinator types (eg. Levin et al. 2001; Steenhuisen et al. 2012)).

The clustering of floral scent chemicals was unlikely explained solely by differences in emission rate or quantity of chemicals making up a floral scent: though they were was an effect of pollinator on both, they did not show significant pairwise differences between pollinator types in most cases. The importance of the identity, ratio, and abundance of volatiles making up the floral scent is consistent with physiological studies showing that particular mixtures of volatiles emitted from flowers, rather than individual constituents, are often necessary to elicit pollination behavior (Riffell et al. 2009a; Riffell et al. 2009b; but see Klahre et al. 2011). The patterns we found in floral scent chemistry recapitulated results from other systems: bird-pollinated flowers produced fewer chemicals and had lower emissions than their ancestors and than other pollination groups (Knudsen et al. 2004; Steenhuisen et al. 2012); bat-pollinated flowers were characterized by the production of novel compounds, which made them cluster in a distinct space from others (Knudsen and Tollsten 1996).

Floral scent chemistry alone also enabled prediction of pollinator better than chance, providing additional evidence that pollinator-mediated convergent evolution has shaped floral scent in this system and further contradicting the historical challenge that pollination syndromes cannot effectively predict pollinator (Rosas-Guerrero et al. 2014). Though pollinator prediction using scent chemistry had higher error than morphology and individual components of scent were not better predictors of pollinator than morphological traits, the ability to predict the value of a flower from its scent remains suggestive of its biologically relevance: in a naturalistic context visual cues many not be as useful or accessible as olfactory cues to some pollinators (eg. Dornhaus and Chittka 2004; Brodmann et al. 2012; Muchhala and Serrano 2015), or both

olfactory and visual cues may be needed to prompt pollination behavior or to facilitate the location of a flower (Grison-Pigé et al. 2002; Raguso and Willis 2002; Kessler et al. 2008; Raguso 2008; Bischoff et al. 2015).

Changes in floral scent were physiologically relevant to pollinators in ways predicted by their observed natural behaviors

Pollinator sensory biases have been shown in other systems to drive the evolution of floral scent (Schiestl and Dötterl 2012), and both the innate and learned olfactory preferences have been shown to play a critical role in the foraging choices of pollinators (Dornhaus and Chittka 2004; Riffell et al. 2008; Dötterl and Schiestl 2012). Changes in floral scent have been documented to mediate pollinator transitions, both alone (Shuttleworth and Johnson 2009; Shuttleworth and Johnson 2010) and in tandem with morphological changes (Klahre et al. 2011; Byers et al. 2014b), and as well as to have direct effects on plant fitness (Majetic et al. 2009; Parachnowitsch et al. 2012b). Multiple lines of evidence in this study suggested that differences in floral scent may be relevant to pollinators in ways material to pollinator-mediated speciation; we gathered evidence that: (a) functional groups of pollinators perceive scents differently, (b) species belonging to the same functional group perceive scents more similarly than do animals belonging to other functional groups, and (c) pollinators can smell these flowers and that differences in pollinators' physiological responses are consistent with differences in observed pollination behavior.

First, our evidence suggests that pollinators from different functional groups may respond to floral scents differently. Antennal responses to individual compounds were distinct in *B. impatiens* (bumble bee) and *M. sexta* (hawk moth), consistent with the distinct innate olfactory

preferences of these insects (Riffell et al. 2008; Dötterl and Schiestl 2012). These insects also appeared to exhibit responses to distinct sets of chemical constituents that made up the tested *Passiflora* floral scents, suggesting that these insects may experience significantly different perceptions of the same floral scent mixture. Second, limited testing suggested that pollinators from the same functional group may process odor mixtures more similarly than members of other functional groups: *Xylocopa* (carpenter bee) and *B. impatiens* responded to similar subsets of compounds making up *P. quadrangularis* scent, while *M. sexta* responded to a largely distinct subset of compounds (see SI Figs. 4,5; **SI doc t30, t32**). Third, responses to floral odors differed in ways consistent with natural observed visitation behavior: tested pollinators tended to show larger responses to scents from *Passiflora* flowers pollinated by their own functional group than to scents from species pollinated by other functional groups, while tested herbivores did not.

Floral morphology and floral scent evolution were correlated

Though floral cues have often been studied independently and in some cases changes in floral scent alone has been sufficient to mediate pollinator transitions (Shuttleworth and Johnson 2010), floral scent often acts in synergy with the visual display of the flower to gate pollination behavior (Raguso and Willis 2002) or learning (Kunze and Gumbert 2001). In this study, floral morphology and floral scent chemistry were significantly and positively correlated with one another at the species level without explicitly taking pollinator into account in the analysis. This correlation was of a comparable magnitude after taking phylogeny into account, suggesting that relatedness did not drive the observed relationship between chemical and morphological traits. After taking pollinator into account, the correlation between morphology and chemistry disappeared, implying that pollinator may be driving the relationship between chemical and

morphological traits. This suggests that floral scent chemistry evolved in tandem with floral morphology to attract particular groups of pollinators, consistent with recent evidence that pollinators prefer, and may learn and perceive, trait combinations in a non-additive manner, with strong interaction effects, suggesting that pollination syndromes likely evolved via selection on trait combinations rather than individual traits (Leonard et al. 2011; Fenster et al. 2015).

Although correlation between traits suggested pollinator-mediated selection, this correlation was not explained by phylogenetic relatedness. We could not explicitly exclude the effect of phylogeny in multivariate clustering analyses against pollinator for chemistry alone or morphology alone, or from the tests showing significant differences between clusters. However, when morphological and chemical data are plotted by monophyletic subgenus, a rough proxy for phylogenetic relatedness, subgenus clusters do not resemble morphological or chemical clusters as would be predicted if relatedness were driving these similarities; rather, subgenus clusters span entire regions of the clusters defined by pollinator type (SI Fig. 3). Furthermore, ancestral state reconstruction confirmed that species representing many independent transitions from across the phylogeny were included, making it still more unlikely that the observed trait clustering by pollinator was driven by relatedness. We did observe a bias in where in the phylogeny certain types of transitions occurred; for example, all transitions to wasp occurred in subgenus *Decaloba* and most transitions to bat occurred in subgenus *Passiflora*, but even within these subgenus clusters, pollinator clusters still appeared to exist (eg, Fig. 3; SI Fig. 3).

Summary and Future Directions

Though past studies have included qualitative chemical data in broad characterizations of pollination syndromes and others have quantitatively characterized floral scent across one or a

few pollinator transitions or for plants chosen as prototypical examples of pollination by a particular pollinator type, we have demonstrated a quantitative floral scent component to pollination syndromes across many evolutionary transitions and pollinator types using chemical, morphological, and phylogenetic evidence from flowering plants and complementary sensory data from pollinators.

The evidence we present for a scent component to pollination syndromes invites rich avenues for future research. To confirm the generality of our findings, quantitative analysis of floral scent chemistry must be conducted in other angiosperm genera with many known pollinator transitions, in tandem with morphological analyses. In particular, this should be examined across a temperate genus; though floral scent has been shown to be a potentially important mediator of pollinator preference across single transitions in temperate plants (Mant et al. 2005; Byers et al. 2014b), tropical groups like the one in our study have been shown to have a stronger relationship between floral morphology and most effective pollinator (Rosas-Guerrero et al. 2014). The mechanism by which floral scent and morphology converge and may evolve together should also be investigated more deeply, especially whether pleiotropy or linkage contribute to the genetic underpinnings of coordinated evolution between these interacting sets of floral traits (Smith 2016); families of chemicals common in floral scent share biochemical pathways with common floral pigments (Zuker et al. 2002; Tholl and Lee 2011; Maeda and Dudareva 2012), allowing the possibility that single gene changes could mediate the evolution of both floral color and floral scent. Additionally, future studies should endeavor to test whether pollinators can detect the floral cues in question and whether they elicit pollination behavior (Schiestl and Ayasse 2002; Riffell et al. 2009a; Shuttleworth and Johnson 2010; Byers et al. 2014b; Muchhala and Serrano 2015) to validate that floral traits are truly evolving in response to

pollinator preference. With floral scent this may be particularly important as a way to disambiguate chemicals used to attract pollinators and those with other purposes, such as plant defense (Kessler and Baldwin 2001; Kessler et al. 2008; Junker et al. 2011), which may be subject to differing selective pressures.

AUTHOR CONTRIBUTIONS

M.R.C. and J.A.R. conceived the study. M.R.C., J.H.C., and J.A.R. designed the experiments. M.R.C., J.H.C., N.C., A.L., N.W., and Y.Y. performed experiments and gathered data. M.R.C. and J.H.C. analyzed the data. M.R.C. wrote the manuscript. M.R.C., J.H.C., Y.Y., and J.A.R. edited the manuscript.

ACKNOWLEDGEMENTS

We thank J. MacDougal and L. Gilbert for helpful discussions and providing botanical samples and pollinator observations; S. Buchman for providing *Xylocopa* specimens; C. Lahondere, C. Vinauger, Y. Hussain, S. Agrawal, and K. Byers for helpful comments on earlier manuscript drafts; R. Olmstead for molecular analysis; D. Giblin and M. Yousoufian for help with voucher specimens; J. Nahum, E. Grason, and D. Vilhena for assistance with statistical analyses; L. Galetto, I. Sazima, M. Sazima, N. Muchhala, S. Krosnick, D. Taylor, M. Huft, A. de Souza, and M. Taylor/Warren Photography for pollinator photographs; K. Lungberg, E. Kim, J. Eom, A. Stone, E. Sanders, and G. Wisswaesser for their aid with plant maintenance, scent collection, and electrophysiological experiments; and D. Ewing, J. Milne, N. Kurashige, P. Beeman, and the UW Greenhouse staff for *Passiflora* care and fruitful discussion. We acknowledge the support of the National Science Foundation under grants DGE-0718124 (M.R.C.) and IOS-1354159

(J.A.R.); the National Institute of Health under grant R01DC013693 (J.A.R.); Riddiford-Truman Fellowship (M.R.C.); UW BEACON student grant (M.R.C.); an Endowed Professorship for Excellence in Biology (J.A.R.); The Washington Research Foundation; and the Human Frontiers in Science Program under grant HFSP-RGP0022 (J.A.R).

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FIGURE LEGENDS

Figure 1: Evolution of pollination in the *Passiflora*

(A) Ancestral state reconstruction using only pollination observations (n = 55) confirms the ancestral state of bee-pollination, as well as the many transitions from one pollinator to another within the genus. An ancestral state reconstruction including 132 species whose major pollinator was inferred by morphology echoed these results (see SI Fig. 2).

(B-D) Different types of pollinators visiting *Passiflora* species

(B) Bees on *Passiflora*: Bee-pollination is the ancestral state in the *Passiflora*; two of the most commonly-pollinating bee genera are the bumble bees (*Bombus*) and the carpenter bees (*Xylocopa*), though other genera of large and small bee have been noted to pollinated differently sized bee-flowers in the *Passiflora*.

Left: Bumble bee *Bombus impatiens* on *P. incarnata*, Wisconsin, USA (photo: D. Taylor). *Bombus impatiens* is the model bee used for electrophysiology in this study.

Right: Carpenter bee *Xylocopa frontalis* on *P. edulis*, Brazil (photo: A. De Souza). A congener, *X. californica arizonenensis*, was used for validating electrophysiology in this study.

(C) Birds on *Passiflora*: In the New World, bird pollinators of *Passiflora* are primarily hummingbirds, but other nectivorous birds, like honeyeaters, visit *Passiflora* where they occur in the Old World.

Left: Hummingbird on *Passiflora* species, Costa Rica (photo: M. Taylor/Warren Photographic).

Right: Eastern spinebill on *P. herbertiana*, Queensland, Australia (photo: S. Krosnick).

(D) Bats on *Passiflora*: Bat-pollination is relatively rare in the *Passiflora*, but has evolved throughout the phylogeny.

Left: *Anoura fistulata* on *P. unipetala*, Ecuador (photo: N. Muchala).

Right: *Anoura caudifer* on *P. ovalis*, Brazil (photo: I. Sazima).

(E) Wasps on *Passiflora*: Wasp-pollination is only known to have evolved in the large subgenus, *Decaloba*, with its relatively small flowers. Nectaring solitary potter wasps have been noted to visit, but so have social vespids and others (see SI Table 4).

Left: Social vespid *Polybia ignoblis* on *P. suberosa*, Argentina (photo: L. Galetto).

Right: *Polistes* species on *P. suberosa*, Brazil (photo: I. and M. Sazima).

Figure 2: Morphological convergence in flowers of the *Passiflora*.

(A) Floral morphology by pollinator: Multivariate clustering (PCO using Gower's dissimilarity) illustrates floral morphology clustering and separating strongly by pollinator, suggesting convergent evolution. Filled symbols are used for species with

direct pollinator observations; open symbols are used for species where quantitative morphology was used to infer the most likely major pollinator.

(B) Floral morphology by relatedness: The same clustering as (A), but grouped by monophyletic subgenus, illustrates the phylogenetic signal in floral morphology, but shows that the phylogenetic clusters are almost orthogonal to the pollination clusters, suggesting that phylogenetic signal is not responsible for the clustering by pollination that we see.

(C) Pollinator transitions in different clades of *Passiflora*: Related species show dramatically different floral morphologies based on pollinator, here showing species pollinated by bee, bird, bat, wasp, moth, and multiple pollinators (a combination of at least two of: bee, bird, butterfly). *Passiflora* species shown, left to right and up to down: (row 1, subgenus *Decaloba*) *P. holosericea, P. aurantia, P. membranacea;* (row 2, subgenus *Decaloba*) *P. adenopoda, P. citrina, P. sanguinolenta, P. lutea, P. capsularis;* (row 3, subgenus *Decaloba) P. standleyi, P. perfoliata, P. murucuja, P. penduliflora, P. boenderi, P. jorullensis;* (row 4, subgenus *Passiflora*) *P. antioquiensis, P. trisecta*, (row 5, subgenus *Passiflora*) *P. amethystina, P. racemosa, P. mucronata, P. mooreana, P. loefgrenii;* (row 6, subgenus *Passiflora*) *P. alata, P. vitifolia, P. setacea, P. coccinea*.

Figure 3: Chemical convergence in the *Passiflora*.

(**A-C**) Chemical clustering in *Passiflora* by pollinator suggests convergent evolution in floral scent: An NMDS on chemical abundance (A, Bray-Curtis), chemical identity (B, Jaccard), and chemical relative ratios (C, Bray-Curtis) by *Passiflora* species shows clustering and separation by pollinator type; for all stress > 0.2 and < 0.25. Note that bat-pollinated species are around the periphery for all clusterings, likely because of the

relatively large number of novel compounds found in these species. Chemical data also showed phylogenetic signal, though less than morphological data, but the clusters formed by subgenera, were very different—almost orthogonal-- from those formed by pollinator type; chemical abundance showed subgenus separation, but relative ratio and identity data had subgenus clusters that were almost indistinguishable (see SI Fig. 3).

(**D**) Differences in the intensity of fragrance: Emission rates were from bee- and bat-flowers were significantly higher than those of bird- and wasp-flowers (24-hour emissions were $\log_{10}$-transformed so that the data would conform to the assumption of equal variance between groups). Emission rate did not explain chemical clustering. Lines show standard error and dots show bounds of the 95% confidence interval.

Figure 4: Model pollinator electrophysiological responses to *Passiflora* scents

Both panels, show the results of a mixed effects model on antennal responses, controlling for differences between individuals. The bars indicate the mean number of standard deviations above the mean that responses to each category of odor were. Lines on each bar indicate standard error (see SI stats tables 11- 13 for 95% confidence intervals of the effect size). The horizontal dashed lines denote the upper limit of the confidence interval for antennal responses to the vegetative odors. Example responses to floral scents of each category are shown for each insect species.

(A) *Bombus impatiens* (n = 9 individuals), the bee model used in electrophysiological experiments, only showed significantly higher responses to scents from bee-pollinated flowers and to the linalool positive control than to vegetative odors according to the 95% CIs.

(B) *Manduca sexta* (n = 8 individuals), the moth model, only showed significantly higher responses to the linalool positive control than to vegetative odors. Only one of the floral scents we were able to include in our odor panel was noted to be visited by moths, but moths were not the primary pollinator for any species of *Passiflora*.

(C) *Heliconius melpomene* (n = 5 individuals), the butterfly herbivore model, also only showed significantly higher responses to the linalool positive control than to vegetative odors.

SUPPLEMENTAL INFORMATION

SUPPLEMENTAL INFORMATION – MATERIALS AND METHODS

Floral Specimens and Plant Rearing

In addition to species already present in the University of Washington botany greenhouse (originally procured from Kartuz Greenhouses), we procured additional seeds and cuttings from John MacDougal, Georgia Vines, Trade Winds Fruits, Hirt's Gardens (via Amazon.com), Grassy Knoll Exotic Plants, and the Passiflora Society International seed bank. After receipt, seeds were germinated by soaking in 10% bleach solution for 5 minutes, rinsing in fresh water, and scoring the surface using nails and fine tweezers before partially embedding in rock wool soaked in fertilized water. A two tank system delivers (a) Plant Marvel Nutriculture General Purpose 20-10-20 and Miller Iron Chelate DP 10% Fe and (b) Plant Marvel Nutriculture Cal-Mag Special 17-5-17 and Canadian Agri Products Magnesium Sulphate Heptahydrate simultaneously into the water stream; the two tank system is necessary to avoid the formation of precipitates. The total nutrient delivery (ppm) is as follows: 100 N, 39.75 P, 100 K, 11.75 Ca, 15.035 Mg, 16.1775 S, 0.185 Cu, 0.275 Zn, 0.275 Mn, 2.41 Fe, 0.11 B, and 0.027 Mo.

About nine seeds were planted in each rose pot. Each pot was placed inside of a clear plastic drinking cup with straw-hole lid (SOLO, Dart Container Corporation, Mason, MI USA) to enhance humidity, and then placed on a heating mat at 23.9 degrees C in the UW Greenhouse (Seattle, WA) to keep the soil warm and increase germination rates. Pots with mold growth were treated with a dusting of cinnamon as an antifungal agent twice per week. Germination occurred within six months of planting, and pots without germinants were discarded after this time. After germination, the plants were acclimatized to lower humidity by opening the straw hole in the top

of the drink cup for one week, then pots were moved out of the drink cups and on to the heating mat. Once the seedlings were large enough to be transplanted into larger pots, they were moved off of the heating mat, and staked for support. They were fed fertilized water twice per day in the rock wool substrate. Plants that were received as seedlings were transplanted into large pots with rock wool upon receipt and fed fertilized water once per day. The plants were kept under a long-day light regime (L:D 15:9 hours) and under the same controlled greenhouse temperature (day low: 16 degrees C, day high: 23 degrees C; night low: 15.6 degrees C, night high: 23 degrees C), except for alpine species *P. trisecta* and *P. mucronata (Sazima and Sazima 1978; Ulmer 2004)*, which were kept in an open air hut outside the UW Greenhouse in Seattle, WA, so that they could be exposed to cooler temperatures (approximately 4.5- 15.5 degrees C) to induce blooming.

Voucher Preparation

For each plant included in the study, a voucher specimen was created for storage at the University of Washington (UW) Herbarium. To create the voucher, fresh plant material, including flowers when available, was collected, pressed between sheets of newspaper, and dried for one week in the UW Greenhouse plant drying oven. Details of plant species, UW Greenhouse accession number, collector, and a unique UW Herbarium accession were recorded and assigned to each specimen (see SI table 1). After drying, specimens were frozen for at least 24 hours, then stored in the Herbarium until they could be mounted and databased by Herbarium staff and volunteers. These vouchers correspond to the UW individuals sequenced for the phylogeny, or, if that plant was unavailable, a sibling from the same seed lot.

Genetic Data and Phylogeny

Sequence data from two plastid loci (*trnT-trnF*, *ndhF*) and two nuclear loci (ITS, *ncpGS*) were used to infer a phylogeny for *Passiflora*. These loci were chosen because they were used in multiple previous phylogenetic studies of *Passiflora* (Muschner et al. 2003; Yockteng and Nadot 2004; Hansen et al. 2006; Muschner et al. 2012; Krosnick et al. 2013; Abrahamczyk et al. 2014) and sequence data for many species were available on Genbank. For all *Passiflora* species for which there was sequence data available on Genbank, Genbank accession numbers were aggregated from the literature (Muschner et al. 2003; Yockteng and Nadot 2004; Hansen et al. 2006; Muschner et al. 2012; Krosnick et al. 2013; Abrahamczyk et al. 2014) and custom software was written in Python by MRC and J. Nahum to scrape sequence data from Genbank. In the following two cases, two Genbank sequences for a species were concatenated to make a more complete sequence for a locus: *trnL* and *trnL-trnF* from Muschner et al 2012 (Muschner et al. 2012) for *trnT-trnF*, and ITS1 and ITS2 from Muschner et al 2003 (Muschner et al. 2003) for ITS.

Additional sequence data for *trnL-trnF*, *ndhF*, and ITS were gathered for 69 species, 66 from living accessions at the UW greenhouse and three from herbarium specimens at WTU. DNA was extracted from leaf tissue, either silica gel-preserved from living specimens or fragments from herbarium specimens, using a modified CTAB protocol, and then purified by isopropanol precipitation. Targeted loci were amplified using a standard PCR protocol with an annealing temperature of 52° C and primers c and f for *trnL-trnF* (Taberlet et al. 1991), 5.5F and 10.2R for *ndhF* (Davis et al. 2001), and ITS4 and ITS5 for ITS (Baldwin 1992). Amplification products were purified by polyethylene glycol precipitation before using as template in Sanger cycle sequencing reactions. The standard Applied Biosystems protocol with BigDye ver. 3.1 was

followed using the primers used for PCR amplification or, where necessary, internal primers d and e for *trnL-trnF* (Taberlet et al. 1991), *Passiflora*-specific 415F-Pass (5'-GAATTCCTTTTAATCAATTTAATCAAGAA-3') and 415R-Pass (5'-TTCTTGATTAAATTGATTAAAAGGAATTC-3') for *ndhF*, and ITS2 and ITS3 for ITS (TJ et al. 1990). Reaction products were filtered through Sephadex G-50 columns before analysis on an Applied Biosystems 3130XL or 3730 Genetic Analyzer (Thermo Fisher Scientific, Grand Island, NY, USA). Sequence data were edited and assembled with Sequencher 4.7 (Gene Codes Corp., Ann Arbor, MI, USA). A full table of Genbank accessions for the genetic data used to create the phylogeny is available in this supplemental material document.

Volatile Headspace (Scent) Collection and Floral Sample Preparation

Between one and six flowers were cut from the plant and placed in beakers of water inside a three liter teflon-lined plastic oven bag (Reynolds, Richmond, VA, USA). These bags were used because they are lined with relatively chemically-inert Teflon, minimizing contamination of the floral bouquet by plastic volatiles as well as chemical reactions between floral headspace volatiles and plastic volatiles. Diaphram pumps were used to pull floral headspace air through adsorbent traps at a flow rate of 2 L/min, capturing the scent volatiles on the surface of adsorbent beads. The traps were created by packing 100 mg of Porapak Q absorbent (mesh size 80–100, Waters Corp., Milford, MA, USA) into cut 7mm borosilicate glass pipettes (VWR Scientific, Brisbane, CA, USA) plugged with silanized glass wool (Restek, Bellefonte, PA, USA). Collections ran for 24 hours (±4 hours) for most specimens to account for any effects of circadian rhythm on scent emission and to enable the characterization of species with low intensity headspace emissions. For each species, headspace was collected from between one and

six genetically-distinct individuals depending on availability over the course of three years of growth in the University of Washington Botany Greenhouse. Three or more samples were collected from most individuals, but for a small number of individuals only one sample was collected due to low flower production. Headspace volatiles were eluted from the adsorbent traps using 800 uL of HPLC-grade hexane (Sigma-Aldrich, St. Louis, MO USA). The samples were stored in 2 mL 9mm borosilicate glass vials with Teflon-lined caps (both vials and caps: Microliter Analytical Supplies, Inc., Suwanee, GA, USA) at -80°C until analysis. A 100 uL aliquot of each sample was concentrated under a stream of nitrogen gas to 8x concentration.

Chemical Analysis with Gas Chromatography and Mass Spectrometry

In order to analyze the chemical composition of the different species of *Passiflora* odor bouquet, three microliters of each concentrated headspace sample was run on a Agilent 7890A gas chromatograph (GC) coupled to a 5975C Network Mass Selective Detector (MS; Agilent Technologies, Palo Alto, CA, USA). The GC was equipped with a DB-5 column (J&W Scientific, Folsom, CA, USA; 30 m, 0.25 mm, 0.25 μm) and used helium as a carrier gas with a flow rate of 1 cc/min^{-1}. The initial oven temperature for each GC run was 50°C for four minutes, followed by a heating ramp of 10°C/min until 250°C was reached; then the oven was maintained at 250°C for 10 minutes to complete the run.

Volatile Headspace (Scent) Analysis

Initial analysis of chromatograms from each GC run was conducted in Agilent ChemStation software (Agilent Technologies). Chromatogram peaks were integrated using ChemStation integrator (parameter values: Initial Area Reject of 1, Initial Peak Width of 0.020, Shoulder

Detection off, and Initial Threshold of 18.0; time values for all parameters were set to 'Initial'). After automatic integration (Agilent ChemStation built-in Integrate method), each chromatogram was assessed by eye and peaks missed by the integrator were added using manual integration. The top five spectral matches for each peak were found using the National Institute of Standards (NIST) mass spectral library (~ 120,000 spectra). A 10ng/uL C7-C30 alkane standard using the same GCMS method was used to calculate Kovats Indices for all peaks; a new alkane standard was run if the column was cut or replaced to ensure that any changes in alkane retention times were accounted for. A custom software written in Python was used to reduce all chemical synonyms from spectrum matching to a single name using synonyms found in the NIST online Chemical WebBook (Linstrom and Mallard 2015), so that name-matching could be performed. Additional software was written to calculate Kovats Index for each peak in each sample, compare it with a database DB-5 Kovats Indices gathered from the literature and from synthetic standards run in the lab (3096 records of plant volatiles; (Arn and Acree 1998; Adams 2001; Skogerson et al. 2011)), and choose the compound as the peak identity that best matched the top 5 spectral matches and had a Kovats Index within 10 units of the same match. A range of ±10 Kovats units was chosen because runs of 41 authentic standards on the lab GCMS always matched Kovats values in the literature within this range. If no match in our Kovats Index database was found, the top spectral match was retained if its match percentage was 40 or above; if not, the peak was discarded. After this process was performed on all samples, data was concatenated to create a single matrix of chemical abundance versus species. Criteria used to combine chemical data for a given species were: (1) if three or more samples of the species were collected: chemical compounds were retained if they were present in 2 or more samples, and the abundance used was the average of all samples for which the compound was present; and (2) If

two or fewer samples of the species were collected: chemical compounds were retained if they were present in 2 or more samples, and the abundance used was the average of all samples for which the compound was present. Compounds present in controls were removed as contaminants (from hexane, GC column bleed, Porapak bleed, etc.), unless the chemical was a known plant volatile, and then the average abundance found in control samples was removed.

Insect Procurement and Rearing

Model insects representative of common pollinators and a *Passiflora* herbivore (bees, hawkmoths, pestiferous butterflies) were used for electrophysiological experiments with *Passiflora* scents. *Manduca sexta* (Lepidoptera: Sphingidae) adults were obtained from the *Manduca*-rearing facility of the Department of Biology of the University of Washington, Seattle. *Heliconius melpomene* (Lepidoptera: Nymphalidae) pupae were imported from Costa Rica through US-based distributor, LPS Imports under USDA APHIS interstate transport permit P526-140108-011 (to MRC and JAR). *Xylocopa californica arizonensis* (Hymenoptera: Apidae) was included because it was the genus of bee most commonly observed to pollinate *Passiflora* in the literature and our personnel observations (see SI Table 4); *Xylocopa* were used to validate the use of *Bombus impatiens* as a bee model in this system. The species *Xylocopa californica arizonensis* was used because of its overlap with Passiflora species in the southwestern USA and its limited availability through a collaborator. Restrictions on the import of bees into the US precluded the use of tropical species of *Xylocopa* common in the pollination literature. *Bombus impatiens* (Hymenoptera: Apidae) were obtained from commercial hive manufacturers (The Green Spot Ltd., Nottingham, NH, USA) and Biobest (Belgium)). *B. impatiens* was chosen because many of the incidences of bee-pollination in the

literature were attributed to *Bombus spp*. in particular (see SI Table 4), and to other large bee generally; because its range overlaps with a large swath of eastern North American *Passiflora* species, some of which it has been recorded visiting (see SI Table 4); because of restrictions on importing bees from the tropics; because the range of *Passiflora* is so large that no single bee species is present across the genus's whole distribution; and because *B. impatiens* is commercially available year-round. For all species, only female insects were used.

Manduca sexta (Lepidoptera: Sphingidae) larvae were reared on artificial diet (modified from (Bell and Joachim 1976)) supplemented with cholesterol (5 g), wheatgerm (144 g; 0.5 mg carotenoids), cornmeal (140 g; 2 mg carotenoids), soy (76 g; 10 mg carotenoids), linseed oil (9 mL), and sugar (36 g). Larvae were reared under long-day light:dark (LD) regimen (LD 17:7) at 25–26 °C and 40–50% relative humidity (RH). Pupae were segregated by sex and held in a rearing room under reverse-photoperiod conditions (LD 14:10) and with a superimposed temperature cycle: LD 26:24 °C. Three days before adult emergence, male pupae were transferred to fiberglass-screen cages (31×31×32 cm) under 75-85% RH and ambient light conditions.

Bombus impatiens (Hymenoptera: Apidae) were obtained from commercial hive manufacturers (The Green Spot Ltd., Nottingham, NH, USA) and Biobest (Belgium)). Hives were provided 1.4 L sugar solution (Biogluc: Biobest, Belgium or an equivalent from The Green Spot Ltd.), supplemented with corn pollen (The Green Spot Ltd., Nottingham, NH, USA) to provide protein for larval development. Bees were kept within the hive box to extend the longevity of the hive to about three months from about one month under free-flying conditions, and to create space for multiple hives in the same facility. The bees were maintained at approximately 30°C under long-day conditions of L:D (18:6). Only worker females were used

for electrophysiological experiments, as this caste likely provides the bulk of pollination services to the hive. Worker females were distinguished from males by their antennae and legs, and from queens by their size and a time of emergence in the colony not concurrent with male reproductives.

Xylocopa californica arizonensis were obtained by S. Buchmann, who wild-caught them in the Sonoran Desert near Tucson, Arizona, USA. They were housed in pop-cap plastic containers with moistened Kimwipes to prevent dehydration. They were maintained at approximately 30° C in dark conditions except during feeding; they were fed a 60% solution of honey and water daily until satiation. Both males and females were used for electrophysiological experiments, as both sexes forage in this solitary group; they were sexed before receipt by S. Buchmann.

Heliconius melpomene pupae were imported from Costa Rica through US-based distributor, LPS Imports under USDA APHIS interstate transport permit P526-140108-011 to MRC and JAR. After arrival, the pupae were sexed (as in (Beebe et al. 1960), LE Gilbert pers. comm.) and then taped by the ends of their abdomens to snap-close cup lids with no straw hole (SOLO, Dart Container Corporation, Mason, MI USA), and enclosed in a cup with a moist paper towel until eclosion; if pupae did not emerge after two weeks or showed fungal growth or presence of parasites, the cups were autoclaved without opening. After emergence, butterflies were kept sex-segregated in parasitoid-resistant mesh flight cages and fed with Gatorade sports beverage soaked into a paper towel (PepsiCo, Purchase, NY, USA) sprinkled with crushed corn pollen (The Green Spot Ltd.), replaced daily (pers. comm. Richard Cowan, LPS LLC).

Electrophysiology and Olfactory Stimulus Presentation

Electroantennograms (EAGs) and gas-chromatograph-coupled electroantennogram detection (GC-EADs) were performed using apparati and procedures as in (Byers et al. 2014). Sample sizes for EAGs were: 8 *M. sexta*, 9 *B. impatiens*, 5 *H. melpomene* individuals.

For GC-EADs, a GC-FID (Agilent 7820A GC with Flame Ionization Detector, Agilent Technologies; DB5 column, J&W Scientific, Folsom, CA, USA) was used with a glass y-splitter was used to present half of the GC effluent to the antenna(e) as the chemical stimulus, to allow half to proceed to the FID detector; chemicals were presented in the order and concentration that the emerged from the GC. For EAGs, chemical stimuli were presented using an odor cartridge, constructed from a 2 mL glass syringe (Air-Tite Products Co., Virginia Beach, VA, USA) and standard 20G 1 inch needle (PrecisionGlide; Becton, Dickinson, and Company; Franklin Lakes, NJ, USA) connected to a piece of plastic tubing with teflon tape to affix it to the stimulus line. All stimuli were pipetted onto a small piece of filter paper (Whatman Inc., Clifton, NJ, USA) in the odor cartridge. All floral stimuli were presented at a concentration equivalent to 100 µL of one flower sampled over 24 hours and eluted into 800 µL of HPLC-grade hexane solvent (Sigma-Aldrich). For individual chemical experiments, each compund was diluted in mineral oil to a partial pressure of 0.1 Torr, to normalize the airborne concentration presented to the insect antenna(e) during stimulation; 5 uL of each solution was used. If the vapor pressure of the undiluted compound was less than 0.1 Torr, 5 uL neat was used. The odor cartridge was connected to its own line with air flowing at 10 mL/min; stimulus presentation was controlled with a solenoid activated by OpenEx software. Responses were recorded to each stimulus and analyzed in R.

Controls: We controlled for responses to plant odors not involved in pollination by presenting 80 µL of each vegetative sample to the insects' antenna(e). Vegetative sample

consisted of all of the leaves on a length of vine between 60 and 110 cm in length sampled for 24 hours before eluting into 800 µL of hexane. Additional negative controls consisted of 100 µL hexane, to control for the amount of hexane in the floral and vegetative samples, and 5 µL mineral oil, which does not volatilize and should control for any antennal response solely to mechanical stimulation from the puff of air accompanying stimulus delivery.

Positive controls consisted of 5 µL (±)linalool, a common floral volatile known to elicit large antennal responses in both moths and bumblebees (see SI figure 5A; eg. *M. sexta*: (Fraser et al. 2003); *B. impatiens*: (Kubo and Ono 2014); *H. melpomene*: (Andersson and Dobson 2003)). Antennae were used only if the preparation was responsive to (±)linalool. Controls were presented at the beginning and end of the experiments, and between the floral and vegetative odor sets.

Plant Scent Stimuli: 30 *Passiflora* species were used for floral scent, depending on availability, and six species were used for leaf scent in the odor panel. Within the floral and vegetative odors sets, odors were presented randomly. Flowers from species that bloomed often and that represented multiple transitions to pollination by a given guild were chosen from our collection for inclusion in the floral stimulus panel. Because of availability, we were not able to use the same number of plant species to represent flowers pollinated by different pollination guilds (see SI Tables 1, 3, and 4).

Insect antennae were prepared by dissecting them from the insect and removing the distal tip with tenotomy scissors. For *M. sexta*, a single antenna was used for each preparation, while for *B. impatiens* two antennae/prep were used to increase signal. The antenna(e) were inserted into Spectra 360 electrode gel-filled (Parker Labs, Fairfield, NJ, USA) glass pipette tips between two silver chloride electrodes constructed using silver wire stripped of its teflon coating (bare:

0.0050", A-M Systems, Sequim, WA, USA) so that the electrodes could measure electrical activity moving across the antenna(e) (as in (Andersson and Dobson 2003; Fraser et al. 2003)). The electrodes were connected to a headstage (A-M systems), chained into a 1800 AC amplifier (A-M Systems), then to a noise reducing device (Humbug Noise Eliminator; A-M Systems), and then into an RZ2 amplifier (Tucker-Davis Technologies, Alachua, FL, USA) before being processed by OpenEx software (Tucker-Davis Technologies). The electrodes and antenna(e) were placed in front of a continuous stream of air (100 mL/min flow; Gilmont flowmeter, Gilmont Industries/Barnant Company, Barrington, IL, USA) at room temperature.

Programming for Data Gathering, Data Cleaning, Statistical Analysis

The following R (Team 2015) packages were used in scripts to conduct:

Comparative analyses, organization of phylogenetic data sets, and plotting of phylogenies: phytools (Revell 2012), geiger (Harmon et al. 2008), ape (Paradis et al. 2004), caper (Orme et al. 2013), and picante (Kembel et al. 2010).

Mixed Models: nlme(Pinheiro et al. 2016)

Multivariate analyses, ordination, and Mantel tests: ecodist (Goslee and Urban 2007), vegan (Okansen et al. 2014) , labdsv (Roberts 2015), cluster (Maechler et al. 2014).

Machine learning: randomForest (Liaw and Wiener 2002).

Other analyses and data cleaning or organization: car (Fox 2011), lsmeans (Lenth and Hervac 2015), dunn.test (Dinno 2015), plyr (Hadley 2011).

Additional figure generation: pheatmap (Kolde 2015), dendextend (Galili 2015), RColorBrewer (Neuwirth 2011).

The following Python (Foundation) packages were used in scripts to:

Scrape sequence files from Genbank: Biopython (Cock et al. 2009)

Convert chemical names into synonyms from NIST webbook (Linstrom and Mallard 2015), check against Kovats indices, and aggregate chemical data into a matrix: BeautifulSoup 4 (Richardson 2015), csv, glob, os, re, urllib, urllib2 (Foundation), requests (Reitz et al. 2015).

SUPPLEMENTAL INFORMATION – RESULTS AND DISCUSSION

Verifying key assumptions: Differences and similarities between model pollinators in response to individual chemical stimuli from floral odors

To determine whether differences in the emission and composition of scent emitted by flowers influences pollination, it was necessary to confirm that different types of pollinators respond to individual chemicals differently. To test this, we presented our model insects with a diverse panel of individual odorants that are commonly found in flowers pollinated by different insects. Aggregate responses by *M. sexta* and *B. impatiens* to the panel of individual compounds were significantly different from one another (PERMANOVA, normalized responses: $R = 0.36$, $p < 0.001$, *M. sexta* n = 9, *B. impatiens* n = 10); confirmed by k-means clustering, with *B. impatiens* and *M. sexta* responses clustering separately with 95% confidence, with the exception of a single *B. impatiens* response falling very slightly outside the 95% confidence region, see SI figure 5).

Our results showed that *B. impatiens* produced similar responses to other *Passiflora* bee pollinators by performing GC-EADs on *Xylocopa californica arizonensis* with fragrant bee-pollinated flower, *P. quadrangularis*. The genus *Xylocopa*, or large carpenter bees, are the group that has been most commonly observed to visit and effectively pollinate bee-pollinated *Passiflora* species (see SI table 4; (Ulmer 2004)). *B. impatiens* (n = 7) and *X. californica* (n = 7)

responded to most of the same compounds within each presented *Passiflora quadrangularis*, in particular, oxygenated terpenes and oxygenated aromatics. In contrast, fewer compounds appeared to be bioactive in *M. sexta* (n = 4).

We confirmed that our model pollinators responded differently to odorants at the concentration they are found in the *Passiflora* flowers themselves, and not just in synthetic odors at a normalized concentration, by performing GC-EAGs on *B. impatiens* and *M. sexta* with the floral scents of *Passiflora* species pollinated by these two guilds *(P. quadrangularis, P. mooreana;* bee, and moth + bee respectively). Antennal deflections were counted as responses for an insect-plant pair if they were 1.5-2.5 standard deviations above mean antennal activity (the threshold was hand-individually calibrated based on differing levels of signal and noise in each preparation), occurred within an 4 second window of a GC peak elution, and if such a deflection occurred in 2 or more preparations. *Bombus impatiens* and *M. sexta* responded to largely different sets of compounds within the same floral scents (*B. impatiens* + *P. quadrangularis* n= 7; *B. impatiens* + *P. mooreana* n = 4; *M. sexta* + *P. quadrangularis* n = 4; *Manduca* + *P. mooreana* n = 4). *Manduca sexta* responded to a larger number of compounds in the moth-visited flower than did *B. impatiens* and *X. californica* and *B. impatiens* responded to a larger number of compounds in the bee-visited flower than did *M. sexta*.

To understand if taking sensory filters into account improves discrimination between the floral scents of flowers pollinated by different pollinator types, we repeated the individual chemical multivariate analyses integrating our results from bee GC-EADs and single odorant EAGs. In our GC-EAD experiments, most of the chemicals bees responded to were oxygenated aromatics, monoterpenes, and oxygenated monoterpenes, while in our single odorant EAG experiments, the bees responded most strongly to those chemical classes with the addition of

sesquiterpenes, oxygenated sesquiterpenes, and nitrogen compounds. Interestingly, when we filtered the chemicals included in the multivariate analysis to include only chemicals in these classes, discrimination between bee and other types of flowers were not noticeably different in significance or in explanatory power (bee GC-EAD-filtered individual chemical data ~ inferred pollinator; ANOSIM; overall: R = 0.420, p = 0.001, **SI doc t34**; bee single odorant EAG-filtered individual chemical data ~ inferred pollinator; ANOSIM; overall: R = 0.427, p = 0.001, **SI doc t35**). However, we found that in both cases, the ability to use the chemical data to discriminate between bird- and wasp- flowers was lost and the ability to discriminate between bird-bat and bat-wasp flowers was gained. This suggests that the chemicals that were excluded, notably alkanes and oxygenated alkanes, are major contributors to the chemical differences between bird-bat and bat-wasp flowers.

SUPPLEMENTAL INFORMATION - TABLES

SI Table 1: Herbarium Voucher and Genbank Accession Information

SPECIES	VOUCHER	GREENHOUSE ACC #	NUC-ITS	CP-NDHF	CP-TRNT-F
ACTINIA	WTU:Clifford 39	5130	Passiflora.actinia_ITS12_AY032832	Passiflora.actinia_2015.3	Passiflora.actinia_trnL_DQ123065_trnL_trnF_AY032767
AFF. GIBERTII	WTU:Clifford 33	PSI-6A	Passiflora.sp.aff.giberti_2014.218	Passiflora.sp.aff.giberti_2014.218	Passiflora.sp.aff.giberti_2014.218
AFF. RUGOSISSIMA	WTU:Clifford 75	6212	Passiflora.rugo.aff_2015.9	Passiflora.rugo.aff_2015.9	Passiflora.rugo.aff_2015.9
ALATA	WTU:Clifford 40	5125	Passiflora.alata_02.91	Passiflora.alata_02.91	Passiflora.alata_02.91
AMBIGUA	WTU:Clifford 41	5162	Passiflora.ambigua_2015.4	Passiflora.ambigua_2015.4	Passiflora.ambigua_trnL_DQ123068_trnL_trnF_DQ123503
AMETHYSTINA	WTU:Clifford 23	5133	Passiflora.amethystina_ITS12_AY102347	Passiflora.amethystina_02.90	Passiflora.amethystina_02.90
ANTIOQUIENSIS	-	15A	Passiflora.antioquiensis_2015.5	Passiflora.antioquiensis_ndhF_KM014391	Passiflora.antioquiensis_trnL_KM014430
ARIDA	WTU:Wiggins 15619	-	Passiflora.arida_03.70	Passiflora.arida_03.70	Passiflora.arida_03.70
AZULITENSIS	-	6330-E	Passiflora.azuliten sis_2014.213	Passiflora.azulitensis_2014.213	Passiflora.azuliten sis_2014.213
BICUSPIDATA	-	PSI-20A	Passiflora.bicuspidata_2014.215	Passiflora.bicuspidata_2014.215	Passiflora.bicuspidata_2014.215

BIFLORA	WTU:Clifford 64	5159	Passiflora.biflora_ 03.71	Passiflora.biflora_0 3.71	Passiflora.biflora_ 03.71
BOENDERI	WTU:Clifford 13	5124	Passiflora.boender i_02.142	Passiflora.boenderi _02.142	Passiflora.boender i_02.142
BOGOTENSIS	WTU:Clifford 86	PSI-10Z	Passiflora.bogoten sis_2014.216	Passiflora.bogotensi s_2014.216	Passiflora.bogoten sis_2014.216
BRYONIOIDES	WTU:Clifford 35	PSI-16B	Passiflora.bryonioi des_nrITS_JX4707 96	Passiflora.bryonioid es_2014.233	Passiflora.bryonioi des_trnL_trnF_JX4 70869
CAERULEA	WTU:Clifford 91	5148	Passiflora.caerulea _02.131	Passiflora.caerulea_ 02.131	Passiflora.caerulea _02.131
CINCINNATA	WTU:Clifford 27	5117	Passiflora.cincinna ta_03.75	Passiflora.cincinata _03.75	Passiflora.cincinna ta_03.75
COCCINEA	WTU:Clifford 95	5141	Passiflora.coccine a_03.76	Passiflora.coccinea_ 03.76	Passiflora.coccine a_03.76
COLINVAUXII	-	1338	Passiflora.colinvau xii_2014.258	Passiflora.colinvauxi i_2014.258	Passiflora.colinvau xii_2014.258
COLOMBIANA	WTU:Clifford 53	5172	Passiflora.colombi ana_03.74	Passiflora.colombia na_03.74	Passiflora.colombi ana_03.74
CORIACEA	WTU:Clifford 92	5151	Passiflora.coriacea _02.105	Passiflora.coriacea_ 02.105	Passiflora.coriacea _02.105
COSTARICENSIS	WTU:Clifford 52	5175	Passiflora.costarec ensis_03.81 (02.81)	Passiflora.costarilen sis_03.81	Passiflora.costaric ensis_03.81
DELTOIFOLIA	WTU:Clifford 69	5155	Passiflora.deltoifol ia_2014.221	Passiflora.deltoifoli a_2014.221	Passiflora.deltoifol ia_2014.221
FOETIDA	WTU:Burnha m 295	-	Passiflora.foetida_ 03.69	Passiflora.foetida_0 3.69	Passiflora.foetida_ 03.69
GIBERTII	WTU:Clifford 19	5135	Passiflora.giberti_ 2014.217	Passiflora.giberti_2 014.217	Passiflora.giberti_ 2014.217
INCARNATA	WTU:Clifford 46	26A	Passiflora.incarnat a_ITS12_AY03283 0	Passiflora.incarnata _2015.6	Passiflora.incarnat a_trnL_DQ123080

					_trnL_trnF_AY032768
LANCETILLENSIS	WTU:Clifford 56	5129	Passiflora.lancetillensis_02.141	Passiflora.lancetillensis_02.141	Passiflora.lancetillensis_02.141
LAURIFOLIA	WTU:Clifford 25	5160	Passiflora.laurifolia_03.78	Passiflora.laurifolia_03.78	Passiflora.laurifolia_03.78
LIGULARIS	WTU:Clifford 73	PSI-8B	Passiflora.ligularis_2014.214	Passiflora.ligularis_2014.214	Passiflora.ligularis_2014.214
LINDENIANA	WTU:Clifford 90	PSI-28B	Passiflora.lindeniana_2015.7	Passiflora.lindeniana_2015.7	Passiflora.lindeniana_trnL_DQ123024_trnL_trnF_DQ123490
LUTEA	-	6326	Passiflora.lutea_2014.232	Passiflora.lutea_2014.232	Passiflora.lutea_2014.232
MALIFORMIS	WTU:Clifford 31	5185	Passiflora.maliformis_03.80	Passiflora.maliformis_03.80	Passiflora.maliformis_03.80
MANICATA	WTU:Clifford 26	5168	Passiflora.manicata_03.79	Passiflora.manicata_03.79	Passiflora.manicata_03.79
MENISPERMIFOLIA	WTU:Clifford 71	5177	Passiflora.menispermifolia_2014.231	Passiflora.menispermifolia_2014.231	Passiflora.menispermifolia_2014.231
MEXICANA	-	1339	Passiflora.mexicana_2014.259	Passiflora.mexicana_2014.259	Passiflora.mexicana_2014.259
MIERSII	WTU:Clifford 17	5171	Passiflora.miersii_ITS12_AY102350	Passiflora.miersii_2015.8	Passiflora.miersii_trnL_DQ123085_trnL_trnF_AY102395
MISERA	WTU:Clifford 61	5115	Passiflora.misera_02.126 (02.106)	Passiflora.misera_02.126 (02.106)	Passiflora.misera_02.126 (02.106)
MOLLISSIMA	-	TWF-G	Passiflora.mollissima_2014.219	Passiflora.mollissima_2014.219	Passiflora.mollissima_2014.219
MOOREANA	WTU:Clifford 7	5139	Passiflora.mooreana_02.136	Passiflora.mooreana_02.136	Passiflora.mooreana_02.136

MORIFOLIA	WTU:Clifford 21	5134	Passiflora.morifolia_02.132	Passiflora.morifolia_02.132	Passiflora.morifolia_02.132
MUCRONATA	WTU:Clifford 65	5145	Passiflora.mucronata_ITS12_AY210951	Passiflora.mucronata_2014.223	Passiflora.mucronata_trnL_trnF_AY210979
MURUCUJA	WTU:Clifford 5	5196	Passiflora.murucuja_02.103	Passiflora.mucucuja_02.103	Passiflora.murucuja_02.103
NEPHRODES	WTU:Clifford 9	5127	Passiflora.nephrodes_02.139	Passiflora.nephrodes_02.139	Passiflora.nephrodes_02.139
OERSTEDII	WTU:Clifford 63	5164	Passiflora.oerstedii_02.127	Passiflora.oerstedii_02.127	Passiflora.oerstedii_02.127
ORGANENSIS	WTU:Clifford 66	5193	Passiflora.organensis_02.95	Passiflora.organensis_02.95	Passiflora.organensis_02.95
PERFOLIATA	WTU:Clifford 10	5194	Passiflora.perfoliata_02.104	Passiflora.perfoliata_02.104	Passiflora.perfoliata_02.104
PLATYLOBA	WTU:Clifford 32	5120	Passiflora.platyloba_02.138	Passiflora.platyloba_02.138	Passiflora.platyloba_02.138
PULCHELLA	WTU:Clifford 6	5146	Passiflora.pulchella_02.102	Passiflora.pulchella_02.102	Passiflora.pulchella_02.102
QUADRANGULARIS	WTU:Clifford 55	5118	Passiflora.quadragularis_02.92	Passiflora.quadrangularis_02.92	Passiflora.quadrangularis_02.92
QUADRIGLANDULOSA	WTU:Clifford 94	1341	Passiflora.quandriglandulosa_2014.260	Passiflora.quandriglandulosa_2014.260	Passiflora.quandriglandulosa_2014.260
RACEMOSA	WTU:Clifford 30	5178	Passiflora.racemosa_02.99	Passiflora.racemosa_02.99	Passiflora.racemosa_02.99
SANGUINOLENTA	WTU:Clifford 47	5158	Passiflora.sanguinolenta_02.93	Passiflora.sanguinolenta_02.93	Passiflora.sanguinolenta_02.93
SEEMANNII	WTU:Clifford 67	5144	Passiflora.seemanii_2014.224	Passiflora.seemanii_2014.224	Passiflora.seemanii_2014.224
SERRATIFOLIA	WTU:Clifford 16	5152	Passiflora.serratifolia_02.130	Passiflora.serratifolia_02.130	Passiflora.serratifolia_02.130

SERRATODIGITATA	WTU:Clifford 29	5113	Passiflora.serratodigitata_02.129	Passiflora.serratodigitata_02.129	Passiflora.serratodigitata_02.129
SERRULATA	WTU:Clifford 70	5122	Passiflora.serrulata_2014.225	Passiflora.serrulata_2014.225	Passiflora.serrulata_2014.225
SEXOCELLATA	WTU:Clifford 59	1337	Passiflora.sexocellata_2014.261	Passiflora.sexocellata_2014.261	Passiflora.sexocellata_2014.261
SPRUCEI	WTU:Clifford 24	5126	Passiflora.spruceii_02.140	Passiflora.spruceii_02.140	Passiflora.sprucii_02.140
STANDLEYI	WTU:Clifford 8	5123	Passiflora.standleyi_02.89	Passiflora.standleyi_02.89	Passiflora.standleyi_02.89
SUBEROSA	-	5157	Passiflora.suberosa_ITS12_AY032841	Passiflora.suberosa_02.96	Passiflora.suberosa_02.96
SUBPELTATA	WTU:Clifford 18	5131	Passiflora.subpeltata_02.134	Passiflora.subpeltata_02.134	Passiflora.subpeltata_02.134
TARMINIANA	WTU:Clifford 57	PSI-38C	Passiflora.tarminiana_2014.220	Passiflora.tarminiana_2014.220	Passiflora.tarminiana_2014.220
TELESIPHE	WTU:Clifford 54	5143	Passiflora.telesyphe_02.133	Passiflora.telesiphe_02.133	Passiflora.telesiphe_02.133
TILIIFOLIA	WTU:Clifford 45	5165	Passiflora.tiliaefolia_03.77	Passiflora.tilaeifolia_03.77	Passiflora.tilaeifolia_03.77
TRIFASCIATA	WTU:Clifford 68	5163	Passiflora.trifasciata_2015.10	Passiflora.trifasciata_02.98	Passiflora.trifasciata_02.98
TRISECTA	WTU:Clifford 62	5121	Passiflora.trisecta_02.88	Passiflora.trisecta_02.88	Passiflora.trisecta_02.88
TUCUMANENSIS	WTU:Clifford 15	PSI-2A	Passiflora.tucumensis_2014.228	Passiflora.tucumensis_2014.228	Passiflora.tucumensis_2014.228
TULAE	WTU:Clifford 11	5169	Passiflora.tulae_ITS12_AY102352	Passiflora.tulae_02.135	Passiflora.tulae_02.135
VITIFOLIA	WTU:Clifford 60	5138	Passiflora.vitifolia_02.137	Passiflora.vitiflolia_02.137	Passiflora.vitifolia_02.137
YUCATANENSIS	WTU:Clifford 58	1340	Passiflora.yucatanensis_2014.262	Passiflora.yucatanensis_2014.262	Passiflora.yucatanensis_2014.262

SI Table 2: Floral Morphology

Additional data from (Christensen 1998; Varassin et al. 2001; Ulmer 2004; Jorgensen et al. 2012).

A) Sepals reflexed?
B) Main sepal/petal color
C) Has petal tube?
D) Has red bracts or stems?
E) Main corona color
F) Corona pattern
G) Sex organ description
H) Minimum flower diameter
I) Maximum flower diameter
J) Ratio max. flower diam./sepal length
K) Petal length
L) Corona length max
M) Minimum Corona Number
N) Corona Orientation

PASSIFLORA SPECIES	A	B	C	D	E	F	G	H	I	J	K	L	M	N
ACTINIA	N	white	N	N	violet	stripes	short, facing inward	6	8	2.91	3.25	3.5	4	75
ADENOPODA	N	white	N	N	purple	stripes	short, facing inward	3.5	5.5	2.08	1.15	1.4	1	75
AFFINIS	N	green	N	N	green	dip	short, facing inward	2	2.5	2.27	0.7	1	2	0
ALATA	N	dark red	N	N	violet	stripes	short, facing inward	7	10	2.50	4	3.5	4	75
ALLANTOPHYLLA	N	green	N	N	yellow	all one	short, facing inward	1	1.2	1.85	0.35	0.3	1	0
AMAZONICA	N	pink	N	N	violet	all one	tall, face outward	9	13	3.06	4	0	1	90
AMBIGUA	N	purple	N	N	violet	stripes	short, facing inward	9	14	3.50	3.5	4	3	75

AMETHYSTINA	Y	violet	N	N	violet	banded	short, facing inward	6	10	3.08	3.5	2.15	4	-30
AMOENA	N	pink	N	N	yellow	all one	tall, face outward	3	4	2.67	1.6	0.7	3	0
AMPULLACEA	N	green	Y	Y	white	all one	tall, facing in one direction	4.5	6	2.00	2.5	0.1	1	90
ANASTOMOSANS	N	pink	N	N	white	dip	tall, face outward	2	4	1.78	2.05	0.1	1	90
ANDINA	N	green	Y	N	white	dip	tall, face outward	3	5	1.00	7.5	0.1	1	90
ANTIOQUIENSIS	N	dark red	N	N	violet	all one	tall, face outward	8	12	2.40	5	0.1	2	90
APETALA	N	green	N	N	white	all one	short, facing inward	1.2	2	2.11	0	0.35	1	0
ARBOREA	Y	white	N	N	yellow	all one	short, facing inward	3	5	2.33	2.15	1.1	2	45
ARIDA	N	white	N	N	light purple	banded	short, facing inward	3	4	2.67	1.5	1.5	6	0
AURANTIA	N	pink	N	N	green	all one	tall, face outward	5	8	2.13	2	1.6	1	90
AURICULATA	N	green	N	N	green	dip	short, facing inward	2	2.5	2.38	0.75	1.15	2	0
BICORNIS	N	violet	N	N	yellow	banded	short, facing inward	3	3.5	1.56	1.15	0.8	3	30
BIFLORA	N	white	N	N	yellow	banded	short, facing inward	2.5	3.5	2.59	1.05	0.8	2	45
BOENDERI	N	green	N	N	yellow	banded	short, facing inward	2	2	1.90	0.45	0.4	2	0
BRACTEOSA	N	orange	Y	N	violet	all one	tall, face outward	1.5	2	0.74	1.3	0	1	90
CAERULEA	N	white	N	N	violet	banded	short, facing inward	7	9	3.27	2.9	2	4	0

CAPSULARIS	N	white	N	N	white	all one	short, facing inward	2.5	4	2.16	1.2	1.3	1	45
CINCINNATA	Y	light purple	N	N	violet	stripes	short, facing inward	7	9	2.25	3	3	2	-30
CINNABARINA	N	red	N	N	yellow	all one	tall, face outward	4.5	7	2.33	1.25	0.9	1	45
CIRRHIFLORA	N	yellow	N	N	yellow	all one	short, facing inward	6	8	3.20	2.5	3	3	90
CITRIFOLIA	N	white	N	N	purple	dip	short, facing inward	4	5	2.08	2.4	1.35	4	0
CITRINA	N	yellow	N	N	yellow	all one	tall, face outward	3.5	5.5	2.20	2.35	1.15	1	45
CHRYSOPHYLLA	N	white	N	N	pink	banded	short, facing inward	3	6	3.00	1.6	1.8	5	0
COACTILIS	N	pink	N	N	violet	banded	tall, face outward	11	16	2.50	5.5	0.1	1	90
COCCINEA	N	red	N	Y	purple	all one	tall, face outward	8	12	3.00	3.75	1.25	3	90
COLINVAUXII	Y	white	N	N	purple	banded	short, facing inward	2.5	4.5	2.43	1	0.85	2	45
CORIACEA	N	green	N	N	yellow	banded	short, facing inward	2.5	3.5	3.18	0	0.7	2	45
COSTARICENSIS	N	white	N	N	light purple	dip	short, facing inward	4	5	2.50	1.4	1.25	1	0
CRISPOLANATA	N	pink	N	N	violet	all one	tall, face outward	5.5	7.5	2.83	2.65	0.1	1	90
CUMBALENSIS	N	pink	N	N	purple	dip	tall, face outward	8	10	2.47	4.05	0.1	1	90
CUNEATA	N	white	N	N	yellow	banded	short, facing inward	3.5	4	3.08	0.6	0.4	2	45
CUPRAEA	N	dark red	N	N	dark red	banded	tall, face outward	4.5	5.5	2.75	1.4	0.4	1	120

DELTOIFOLIA	N	light purple	N	N	violet	spots	short, facing inward	6	7	2.00	3.5	2.75	5	-30
DIOSCOREIFOLIA	N	white	N	N	white	banded	tall, facing in one direction	5	6	2.26	1.75	2.8	1	0
DISCOPHORA	N	white	N	N	yellow	banded	short, facing inward	3.5	4	1.67	1.7	1.8	3	30
EDULIS	N	white	N	N	violet	dip	short, facing inward	6	8	3.02	2.35	1.65	4	0
EXURA	Y	light purple	N	N	purple	all one	short, facing inward	8	9	2.25	4	3	NA	-60
FOETIDA	N	pink	N	N	purple	banded	short, facing inward	3.5	5	2.00	2.5	1.5	4	0
GALBANA	N	white	N	N	white	all one	tall, facing in one direction	NA	NA	NA	NA	2	2	-30
GARCKEI	N	light purple	N	N	purple	banded	short, facing inward	7.5	8.5	2.27	3.25	3.25	2	0
GIBERTII	N	white	N	N	purple	stripes	short, facing inward	6	9	2.77	2.45	1.9	5	0
GILBERTIANA	N	green	N	N	yellow	all one	short, facing inward	2	2	1.48	0.3	0.6	1	30
GRACILENS	N	pink	N	N	purple	all one	tall, face outward	2	3.5	2.50	1.4	0	1	90
GUATEMALENSIS	N	white	N	N	yellow	all one	short, facing inward	4	5	3.03	1.6	0.65	2	30
HAEMATOSTIGMA	N	white	N	N	yellow	spots	short, facing inward	4	6	2.40	1.75	1.15	2	0
HAHNII	N	white	N	N	yellow	all one	short, facing inward	4	6	2.55	2.35	3.5	1	45
HARLINGII	N	orange	Y	N	NA	NA	tall, face outward	1	3	0.60	5	0.2	2	NA
HELLERI	N	white	N	N	dark red	banded	short, facing inward	3	3	1.62	1.4	0.65	1	90

HERBERTIANA	N	pink	N	N	yellow	all one	tall, face outward	5	8	2.58	1.85	1.25	1	90
HOLOSERICEA	N	white	N	N	yellow	banded	short, facing inward	3.5	4.5	2.90	1.4	1.1	2	0
INCARNATA	N	white	N	N	purple	banded	short, facing inward	6	8	2.67	3	2	2	0
INDECORA	N	white	N	N	purple	stripes	short, facing inward	3	4	2.96	0.9	0.55	2	0
INSIGNIS	Y	pink	N	N	violet	dip	tall, face outward	13	17	2.45	6.75	0.9	1	120
JAMESONII	N	pink	N	N	violet	all one	tall, facing in one direction	9.5	12	2.31	5.5	0.15	1	90
JORULLENSIS	Y	green	N	N	red	all one	short, facing inward	3.5	4.5	2.25	0.55	1.5	1	-30
KARWINSKII	N	white	N	N	purple	banded	short, facing inward	4	5	2.63	1.15	1.4	1	30
KAWENSIS	Y	white	N	N	yellow	all one	short, facing inward	6	7	1.87	3.5	1	2	75
KERMESINA	Y	dark red	N	N	violet	banded	tall, face outward	8	9.5	1.09	8.75	1.4	5	90
LANATA	N	pink	N	N	purple	all one	tall, face outward	5.5	7.5	2.34	3.2	0	1	90
LANCETILLENSIS	N	white	N	N	yellow	banded	short, facing inward	4.5	5	2.50	2.3	1.75	2	-30
LAURIFOLIA	N	purple	N	N	purple	banded	short, facing inward	5	7	3.11	2.1	3.5	6	45
LEPTOMISCHA	N	pink	N	N	violet	all one	tall, face outward	10	14	2.72	4.45	0	1	90
LIGULARIS	N	white	N	N	purple	stripes	short, facing inward	6	7	2.80	2.5	3	5	75
LINDENIANA	N	white	N	N	yellow	all one	short, facing inward	5	6	2.18	2.25	1.3	3	0

LOBATA	N	white	N	N	white	stripes	tall, facing in one direction	4.5	6	2.11	1.75	1.8	1	0
LOEFGRENII	Y	purple	N	N	violet	banded	tall, face outward	9	12	2.53	4.75	2	6	0
LOXENSIS	N	light purple	N	N	white	all one	tall, face outward	10	14	2.00	7	0	1	90
LUETZELBURGII	N	red	N	N	white	all one	tall, face outward	5	6	2.40	2.5	0.55	3	90
LUZMARINA	N	pink	N	N	purple	dip	tall, face outward	3.5	5	2.04	2.2	0	1	90
MACROPHYLLA	N	white	N	N	yellow	all one	tall, face outward	6	7	1.87	3.6	1.9	2	45
MACROPODA	N	white	N	N	white	all one	tall, facing in one direction	8	12	2.53	4.25	2	4	0
MALIFORMIS	Y	purple	N	N	violet	stripes	short, facing inward	6	7.5	1.88	3.25	3.25	4	75
MANDONII	N	pink	N	N	violet	all one	tall, face outward	7	10	2.35	3.65	0.1	1	75
MANICATA	N	red	N	N	violet	all one	tall, face outward	6	9	2.47	3.25	0.3	3	90
MANSOI	N	white	N	N	yellow	all one	short, facing inward	4.5	4.5	4.29	1.5	1.15	2	30
MATHEWSII	N	pink	N	N	white	banded	tall, face outward	4	5.5	1.77	3.1	0.3	1	90
MEMBRANACEA	N	green	Y	Y	white	all one	tall, face outward	3.5	3.5	0.74	3.75	1.6	1	90
MENISPERMIFOLIA	N	light purple	N	N	violet	stripes	short, facing inward	6	7.5	2.50	3	3	2	0
MEXICANA	Y	green	N	N	pink	all one	short, facing inward	NA	NA	NA	0	NA	3	0
MICROSTIPULA	N	white	N	N	dark red	stripes	short, facing inward	4.5	5	2.44	2.15	1.1	2	30

MIERSII	Y	white	N	N	violet	stripes	short, facing inward	4	5	2.50	2	1.25	4	0
MISERA	Y	white	N	N	white	all one	short, facing inward	NA	NA	NA	NA	NA	2	0
MIXTA	N	pink	N	N	purple	banded	tall,face outward	5	9	2.31	3.75	0	1	90
MOOREANA	N	white	N	N	purple	banded	short, facing inward	4	7	2.46	2.75	1.8	3	0
MORIFOLIA	N	white	N	N	purple	stripes	short, facing inward	2.5	3	1.82	0.9	0.8	1	30
MUCRONATA	N	white	N	N	white	all one	tall, facing in one direction	8	9	2.90	2.65	1	2	45
MURUCUJA	N	red	N	N	red	all one	tall, face outward	4	6	2.67	1.5	1.25	1	90
OBOVATA	Y	white	N	N	light purple	dip	short, facing inward	4	4	2.67	1.6	2.1	2	0
OERSTEDII	N	white	N	N	purple	banded	short, facing inward	4	5.5	2.44	2.25	2.25	2	0
ORGANENSIS	Y	light purple	N	N	purple	banded	short, facing inward	3	4.2	2.80	0.85	0.5	1	45
OVALIS	N	white	N	N	white	all one	tall, facing in one direction	5	6	2.18	1.65	1	2	75
PARRITAE	N	orange	N	N	purple	all one	tall, face outward	10	12	2.11	4.5	0.1	1	90
PEDUNCULARIS	N	white	N	N	white	all one	tall,face outward	7	11	2.59	3.85	0.25	8	90
PENDULIFLORA	N	green	N	N	yellow	all one	tall, face outward	4	4	2.29	2.05	0.35	1	120
PERFOLIATA	N	dark red	N	N	green	all one	tall, face outward	3.5	5.2	3.47	1.8	0.4	1	120
PILOSICORONA	N	pink	N	N	violet	all one	tall, face outward	11	16	2.41	5.5	0.65	3	120

PINNATISTIPULA	N	pink	N	N	violet	all one	tall, face outward	6	10	2.22	4.5	1.45	1	0
PITTIERI	Y	white	N	N	yellow	spots	short, facing inward	5	8	2.46	3.25	1.8	4	0
PLATYLOBA	Y	purple	N	N	violet	stripes	short, facing inward	5	6	2.18	2.25	2.5	2	75
PUNCTATA	Y	white	N	N	purple	banded	short, facing inward	2.5	4.5	2.43	1	0.85	2	45
QUADRANGULARIS	N	light purple	N	N	violet	stripes	short, facing inward	10	12	3.12	3.85	6.5	5	75
QUADRIGLANDULOSA	Y	red	N	N	red	stripes	tall, face outward	9	15	2.94	4.4	0.95	3	75
RACEMOSA	N	red	N	N	white	all one	tall, face outward	8	10	2.50	3.75	0.85	3	75
RECURVA	N	white	N	N	white	all one	tall, facing in one direction	4	5	2.86	1.55	1	2	0
REFLEXIFLORA	Y	pink	N	N	violet	all one	tall, face outward	5	7	2.00	3.5	0.2	2	0
RHAMNIFOLIA	Y	white	N	N	green	spots	short, facing inward	3.5	4	2.29	1.75	1.25	2	45
ROSEORUM	N	violet	N	N	purple	all one	tall, face outward	7	9	2.05	4.2	0	1	90
ROVIROSAE	N	white	N	N	purple	banded	short, facing inward	4	4	1.67	2.2	1.45	2	0
RUBRA	N	white	N	N	pink	banded	short, facing inward	2.8	4.5	3.00	1.1	0.75	1	30
SAGASTEGUII	N	purple	N	N	purple	banded	tall, face outward	3.5	4.8	2.74	1.4	0.55	2	90
SANCTAE-BARBARAE	N	orange	N	N	violet	all one	tall, face outward	7	9	1.61	5.6	0	1	90
SANGUINOLENTA	N	pink	N	N	red	banded	tall, face outward	3.5	5	2.70	1.25	0.55	2	90

SEEMANNII	Y	light purple	N	N	purple	stripes	short, facing inward	8	10	3.08	3	2.8	2	75
SERRATIFOLIA	N	light purple	N	N	violet	dip	short, facing inward	5	7	2.30	2.55	3.25	4	-30
SERRATODIGITATA	Y	light purple	N	N	violet	stripes	short, facing inward	7	9	2.57	2.5	2.75	3	75
SETACEA	N	white	N	N	white	all one	tall, facing in one direction	9	10	2.67	2.25	1	1	0
SEXFLORA	N	white	N	N	purple	banded	short, facing inward	1.8	2.6	2.26	0.85	0.6	2	30
SEXOCELLATA	N	green	N	N	yellow	banded	short, facing inward	2.5	3.5	3.18	0	0.7	2	45
SPECIOSA	Y	red	N	Y	white	all one	tall, face outward	10	12	2.40	5	0.8	2	90
SPHAEROCARPA	N	white	N	N	yellow	all one	short, facing inward	4	5	2.33	1.7	1.2	2	0
SPRUCEI	N	light purple	N	N	purple	stripes	short, facing inward	7	7	2.15	3	1.25	4	0
STANDLEYI	Y	purple	N	N	yellow	all one	short, facing inward	3	4	2.11	1.55	0.85	2	0
SUBEROSA	N	green	N	N	yellow	banded	short, facing inward	1.3	2.5	2.50	0	0.5	2	45
SUBLANCEOLATA	Y	dark red	N	N	white	banded	tall, face outward	7	8	2.29	3.5	1	5	90
SUBPELTATA	N	white	N	N	white	all one	short, facing inward	4	5.5	2.20	2.5	1.75	5	0
TARMINIANA	N	pink	N	N	purple	all one	tall, face outward	8	11	1.76	4.75	0	1	90
TATEI	N	white	N	N	purple	stripes	short, facing inward	3	4	2.58	0.8	0.8	2	0
TELESIPHE	Y	white	N	N	white	all one	short, facing inward	3.5	4.5	2.00	1.2	2.4	2	30

TENUIFILA	N	white	N	N	light purple	banded	short, facing inward	4	5	2.86	1.4	0.6	4	30
TINA	N	white	N	N	dark red	banded	short, facing inward	6	7.5	2.14	3.5	2.4	5	45
TRIFASCIATA	Y	white	N	N	white	all one	short, facing inward	2.5	3.5	2.41	0.8	0.9	2	-30
TRIFOLIATA	N	pink	N	N	violet	all one	tall, facing in one direction	4	5.5	2.00	2.6	0	1	90
TRIPARTITA	N	pink	N	N	purple	banded	tall, facing in one direction	6	9	2.25	4	0	1	90
TRISECTA	N	white	N	N	white	all one	tall, facing in one direction	6	9	2.40	3.5	0.35	3	0
TUCUMANENSIS	N	white	N	N	violet	stripes	short, facing inward	3	5.5	3.24	1.8	1.3	4	30
TULAE	N	pink	N	N	orange	all one	tall, face outward	5.5	7	2.00	2.75	1.75	1	90
UMBILICATA	Y	violet	N	N	violet	all one	tall, face outward	3.5	5	1.54	3.25	0.35	3	45
UNIPETALA	N	green	N	N	green	all one	tall, facing in one direction	NA	NA	NA	3.2	0.1	1	90
VESPERTILIO	N	white	N	N	white	all one	short, facing inward	4	5	2.78	1.1	1.5	2	-30
VIRIDIFLORA	Y	green	N	Y	green	all one	tall, face outward	3	3.5	1.89	NA	0.3	1	90
VITIFOLIA	N	red	N	N	white	all one	tall, face outward	12	16	2.37	6.15	2.2	3	75
XIIKZODZ	N	green	N	N	black	banded	short, facing inward	2	2	1.74	0	0.9	5	-30
YUCATANENSIS	N	white	N	N	yellow	banded	tall, face outward	3	3	1.40	1.65	0.85	2	45

SI Table 3: Pollinator and Visitor Observations

- Online plant database Tropicos was used to track the currently accepted name of *Passiflora* species to compare with those used in the literature. Species are listed under their currently accepted name for all data sets in this manuscript.

PASSIFLORA SPECIES	NAME USED IN PUBLICATION (WITH GENUS PASSIFLORA UNLESS OTHERWISE NOTED)	LOCATION OF OBS.	VISITORS/POLLINATORS	POLLINATOR CLASS	REFERENCE
ACTINIA	*actinia*	Brazil	bee	bee	passionflow.co.uk
ADENOPODA	*adenopoda*	Costa Rica	bees: *Xylocopa* sp, *Epicharis* sp	bee	MacDougal 1984 (Macdougal 1984)
AFFINIS	*affinis*	Texas, USA	wasp	wasp	MacDougal 1984 (Macdougal 1984)
ALATA	*alata*	NA	bird	bird	Grant 1950 (Grant 1950)
ALATA	*alata*	Santa Geneva Municipal Reserve, Campinas, Sao Paulo, Brazil	*Epicharis flava*, Xylocopa brasilianorum*, Acanthopus exellens*, Centris labrosa, Centris*, other medium sized bees that did not contact the reproductive organs	bee	Koschnitzke and Sazima 1997 (Koschnitzke and Sazima 1997)
ALATA	*alata*	South-eastern Brazil	*Centris flavifrons*, Centris longimana*, Centris lutae*, Centris sp*, Xylocopa brasilianorum*, Xylocopa ordinaria*, Eulaema cingulata*, Centris analis, Centris derasa, Euglossa sp, Apis mellifera*	bee	Varassin et al 2008 (Varassin et al. 2001)
ALATA	*alata*	Brazil	*Epicharis flava*, Euglossa cordata, Plebeia sp*	bee	Gaglianone et al 2010 (Gaglianone et al. 2010)
AMETHYSTINA	*amethystina*	Santa Geneva Municipal Reserve, Campinas, Sao Paulo, Brazil	*Xylocopa brasilianorum*, Centris* (does not contact reproductive organs because too small), *Eulaema nigrita* (male, to collect scent from corona; did not contact reproductive)	bee	Koschnitzke and Sazima 1998 (Koschnitzke and Sazima 1997)
AMPULLACEA	*ampullacea*	Ecuador	hummingbird	bird	Escobar 1980 (Escobar 1980)
AMPULLACEA	*ampullacea*	NA	hummingbird	bird	Ulmer and MacDougal 2004 (Ulmer 2004)

AMPULLACEA	*ampullacea*	Ecuador	*Ensifera ensifera*	bird	Abrahamczyk et al 2014 (Abrahamczyk et al. 2014)
ANTIOQUIENSIS	*antioquiensis*	NA	hummingbird	bird	Ulmer and MacDougal 2004 (Ulmer 2004)
APETALA	*apetala*	Costa Rica	small bee	bee	MacDougal 1984 (Macdougal 1984)
ARIZONICA	*arizonica*	NA	moth	moth	Goldman 2003 (Goldman 2003)
AURANTIA	*aurantia*	NA	honey creeper	bird	Krosnick et al 2015 (Krosnick et al. 2015)
BIFLORA	*biflora*	Costa Rica	bee	bee	MacDougal 1984 (Macdougal 1984)
BREVIFILA	*brevifila*	Costa Rica	large bee	bee	(Macdougal 1984)
CAERULEA	*caerulea*	NA	bees: "humble bee", *Xylocopa violacea*	bee	(Knuth 1898)
CAERULEA	*caerulea*	NA	*Xylocopa* (6 species)*, *Bombus tucumanus**, *Centris spp, Erinnyis ello (Sphingidae),* hummingbirds (including *Chlorostilbon aureoventris*)	bee	(Garcia and Hoc 1998)
CAERULEA	*caerulea*	Argentina	*Xylocopa splendidula, Bombus spp*	bee	(Torres et al. 2012)
CAPSULARIS	*capsularis*	Costa Rica	wasp	wasp	(Macdougal 1984)
CAPSULARIS	*capsularis*	Minas Gerais, Brazil	self*	self	(Faria and Stehmann 2010)
CAPSULARIS	*capsularis*	Santa Geneva Municipal Reserve, Campinas, Sao Paulo, Brazil	moth? (open at night, but no visitors observed; however, moth scales found on stigma of several specimens)	moth, self	(Koschnitzke and Sazima 1997)
CHRYSOPHYLLA	*chrysophylla*	NA	*Ptilloglossa spp (Colletidae)*, Thygater analis (Apidae)*, Xylocopa augusti*	bee	(García and Hoc 2001)
CINCINNATA	*cincinnata*	Brazil	*Oxaea flavescens (Andrenidae), Euglossa spp, Augochora spp (Halictidae), Epicharis spp, Apis mellifera*	bee	(Souza and Teresinha 2011)
CITRINA	*citrina*	Honduras	hummingbird	bird	(Macdougal 1989)
COCCINEA	*coccinea*	NA	butterflies: *Heliconius wallacei, Heliconius burneyi*	*Heliconius*	(Benson et al. 1975)
COCCINEA	*coccinea*	Brazil	Phaethornis superciliosus	bird	(Leal et al. 2008)
CORIACEA	*coriacea*	Mexico	hymenopteran	bee or wasp	(Macdougal 1992)
COLOMBIANA	*colombiana*	NA	bat	bat	(Abrahamczyk et al. 2014)
DIOSCOREIFOLIA	*dioscoreifolia*	Costa Rica	bee: *Epicharis sp*	bee	(Macdougal 1984)

EDULIS	*edulis*	Brazil	*Xylocopa*	bee	(Ruggiero et al. 1976)
EDULIS	*edulis*	Brazil	*Acanthopus*, Bombus pauloensis*, Centris (4 sp)*, Epicharis flava*, Eulaema nigrita*, Xylocopa (3sp)**	bee	(Yamamoto et al. 2012)
FOETIDA	*foetida*	NA	*Ptilloglossa spp (Colletidae)*, Thygater analis (Apidae)*, Xylocopa augusti, Pseudaugochloropsis sp (Halictidae*; pollen thieves)	bee	(García and Hoc 2001)
FOETIDA	*foetida var. gossypifolia*	Costa Rica	bees: *Xylocopa sp* (*), *Centris sp (*), Trigona sp.*	bee	Lind 1976 in (Macdougal 1984)
FOETIDA	*foetida, s. lat.*	Mexico, Belize, Costa Rica	bees: *Ptilloglossa spp* (3 spp)	bee	(Janzen 1968a)
FOETIDA	*foetida*	Argentina	*Bombus opifex, Melissoptila sp, Thygater sp*	bee	(Torres et al. 2012)
GALBANA	*galbana*	South-eastern Brazil	*Glossophaginae**	bat	(Varassin et al. 2001)
GILBERTIANA	*gilbertiana*	Mexico	wasp*, hummingbird	wasp	(Macdougal 2004)
HAHNII	*cookii*	Mexico	moth	moth	(Macdougal 1984)
HELLERI	*helleri*	Guatemala	bee	bee	(Macdougal 1984)
HELLERI	*helleri*	Costa Rica	bee	bee	(Macdougal 1984)
HERBERTIANA	*herbertiana*	Australia	Lewin's honeyeater (most frequent), Noisy Miner, Eastern Spinebill	bird	(Krosnick et al. 2015)
HOLOSERICEA	*holosericea*	Mexico	bee	bee	(Macdougal 1984)
INCARNATA	*incarnata*	Oklahoma, USA	bees: *Xylocopa sp (*), Halictidae*	bee	(Hardin et al. 1972)
INCARNATA	*incarnata*	North Carolina, USA	bee: *Xylocopa sp.*	bee	(Macdougal 1984)
INCARNATA	*incarnata*	Texas, USA	bees: *Xylocopa virginica(*), Bombus pennsylvanicaus, Xylocopa micans*	bee	(Frankie and Vinson 1977)
JAMESONII	*jamesonii*	NA	hummingbird	bird	(Ulmer 2004)
JORULLENSIS VAR. SALVADORENSIS	*jorullensis var. salvadorensis*	Mexico	hummingbird (unlikely to pollinate)	bird	(Macdougal 2004)
JORULLENSIS	jorullensis	Mexico	wasp	wasp	(Macdougal 1989)
KERMESINA	*kermesina*	NA	butterfly: *Heliconius ethilla*	*Heliconius*	(Benson et al. 1975)
KERMESINA	*kermesina*	Brazil	*Heliconius ethila* (pollen on wings and antennae; most frequent)*, *Trochilidae sp* (pollen on head; most efficient!)*, other Lepidoptera, *Euglossa cordata, Plebeia sp*	bird	(Benevides et al. 2013)
LANCETILLENSIS	*lancetillensis*	Mexico	1 cm long bee in *Anthrophoridae**, hummingbirds	bee	(Macdougal and Hansen 2003)

LOBATA	*lobata*	Costa Rica	bee: *Epicharis bova*	bee	(Macdougal 1984)
LOEFGRENII	*loefgrenii*	Brazil	hermit hummingbirds, large bees	bird	(Vitta 1997)
LUTEA	*lutea*	Illinois, USA	bees: *Colletes latitarsis (*), Anthemurgus passiflorae* (*); wasps: *Eumenes fraternus, Anacabro sp*	bee	Robertson 1928 in (Macdougal 1984)
LUTEA	*lutea*	North and South Carolina, USA	Eumenid wasp	wasp	(Macdougal 1984)
LUTEA	*lutea*	Texas, USA	*Monobia quadridens* (Eumenid wasp)	wasp	Neff, Jack, pers comm
LUZMARINA	*luzmarina*	NA	hummingbird	bird	(Ulmer 2004)
MALACOPHYLLA	*malacophylla*	Brazil	*Xylocopa frontalis*, Xylocopa ordinaria*, Apis mellifera, Plebeia sp*	bee	(Benevides et al. 2013)
MATTHEWSII	*matthewsii*	Ecuador	bee: *Bombus funebris, Bombus robustus*	bee	(Escobar 1980)
MEMBRANACEA	*membranacea*	Guatemala, Costa Rica	hummingbird	bird	(Macdougal 1984)
MEMBRANACEA	*membranacea*	NA	hummingbird	bird	(Ulmer 2004)
MEXICANA	*mexicana*	Mexico	wasp	wasp	(Macdougal 2004)
MEXICANA	*mexicana*	Mexico	large bee	bee	(Macdougal 1989)
MIERSII	*miersii*	Santa Geneva Municipal Reserve, Campinas, Sao Paulo, Brazil	*Xylocopa* (2 species)*, *Epicharis flava*, Eulaema nigrita**	bee	(Koschnitzke and Sazima 1997)
MISERA	*misera*	NA	*Ptilloglossa spp (Colletidae)*, Thygater analis (Apidae)*, Xylocopa augusti*	bee	(García and Hoc 2001)
MISERA	*misera*	Minas Gerais, Brazil	*Ptilloglossa spp*	bee	(Faria and Stehmann 2010)
MIXTA	*mixta*	Columbia	hummingbird: *Ensifera ensifera*	bird	(Snow and Snow 1980)
MIXTA	*mixta*	Ecuador	bee: *Bombus funebris, Bombus robustus*	bee	(Escobar 1980)
MIXTA	*mixta*	Ecuador	*Ensifera ensifera*	bird	(Lindberg and Olesen 2001)
MOOREANA	*mooreana*	NA	*Xylocopa* (6 species)*, *Bombus tucumanus*, Centris spp, Erinnyis ello (Sphingidae),* hummingbirds (including *Chlorostilbon aureoventris*)	bee, moth	(García and Hoc 2001)
MOOREANA	*mooreana*	Brazil	*Xylocopa ordinaria*, Xylocopa nigrocincta*, Xylocopa augusti* , Centris tarsata*, Centris sp** (day, frequently), *Errinyis ello (Sphingidae*, night; pollen transfer observed on head; nectar concentration	bee, moth	(García and Hoc 1998)

			changed at night to be more acceptable for Sphingids after sunset)		
MUCRONATA	*mucronata*	Brazil	*Glossophaga soricina* (long-tongued)*, *Carollia peripicillata* (short-tongued)*, sphingid and noctuid moths and wasps (night; ineffectual pollinators); day: diurnal bees, wasps, butterflies, and hummingbirds (all ineffectual except *Apis*)	bat	(Sazima and Sazima 1978)
MUCRONATA	*mucronata*	South-eastern Brazil	*Glossophaginae*, Anoura caudifer*, Carollia perspicillata*, Glossophaga soricina**	bat	(Varassin et al. 2001)
MURUCUJA	*murucuja*	NA	hummingbird	bird	(Kay 1998), (Abrahamczyk et al. 2014)
OBOVATA	*obovata*	Costa Rica	bee	bee	(Macdougal 1984)
OVALIS	*Tetrastylis ovalis*	Brazil	bat	bat	(Buzato and Franco 1992)
SUBLANCEOLATA	*palmeri, var. sublanceolata*	NA	birds	bird	Boender 2004, pers obs
PEDUNCULARIS	*peduncularis*	Peru	bat	bat	(Abrahamczyk et al. 2014)
PENDULIFLORA	*penduliflora*	Jamaica	*Monophyllus redmani* (Greater Antillean long-tongued bat; pollen found on mist-netted bats)*, *Apis mellifera, Trochilus polytmus*	bat	(Kay 2001)
PENDULIFLORA	*penduliflora*	Jamaica	moth (mentioned as visitor in addition to bats; species unidentified)	moth	(Kay 1998)
PERFOLIATA	*perfoliata*	NA	hummingbird	bird	(Ulmer 2004)
PLATYLOBA	*platyloba*	Costa Rica	large bee	bee	(Frankie et al. 1983)
POHLII	*pohlii*	Minas Gerais, Brazil	*Ptilloglossa spp (Colletidae; P. dubia, P. styphalspis, P. latecalcarata*)	bee	(Faria and Stehmann 2010)
BICORNIS	*pulchella*	Costa Rica	bee: *Xylocopa gualanensis*	bee	(Baker et al. 1983)
QUADRANGULARIS	*quadrangularis*	Java, South America	bee: *Xylocopa sp.*	bee	(Faegri and van der Pijl 1979)
QUADRANGULARIS	*alata*	Costa Rica	large bee	bee	(Janzen 1968b)
QUADRIGLANDULOSA	*quadriglandulosa*	NA	hummingbird	bird	(Ulmer 2004)
ROSEORUM	*roseorum*	NA	hummingbird	bird	(Lewis 2001; Santos et al. 2014)
SANGUINOLENTA	*sanguinolenta*	NA	hummingbird	bird	(Ulmer 2004)
SANGUINOLENTA	*sanguinolenta*	NA	hummingbird	bird	(Macdougal 1989)
SEXFLORA	*sexflora*	Costa Rica	wasp	wasp	(Macdougal 1984)
SEXOCELLATA	*sexocellata*	NA	*Colletes sp*	bee	(Porter-Utley 2014)

SPECIOSA	*speciosa*	South-eastern Brazil	*Trochilidae*, Phaethornis idaliae*, Apidae, Euglossinae* bees	hummingbird	(Varassin et al. 2001)
SPECIOSA	*speciosa*	NA	hummingbird	bird	(Ulmer 2004)
SUBEROSA	*suberosa*	Minas Gerais, Brazil	wasps (*Polybia ignobilis*), self	wasp, self	(Faria and Stehmann 2010)
SUBEROSA	*suberosa*	Santa Geneva Municipal Reserve, Campinas, Sao Paulo, Brazil	*Mischocyttarus interjectus*, Eumenidae sp**, 4 ant species (thieves), *Plebia droryana* and *Augochorella michaelis* (bees that induce autopollination)	wasp, self	(Koschnitzke and Sazima 1997)
SUBEROSA	*suberosa*	Brazil	*Augochloropsis patens (Halictidae)*, Hypanthidium foveolatum (Megachilidae)*, Plebeia* bees (small)	bee	(Gaglianone et al. 2010)
SUBEROSA	*suberosa*	NA	*Polistes*	wasp	(Porter-Utley 2014), (Koschnitzke and Sazima 1997)
SUBEROSA	*suberosa*	Argentina	*Polybia ignobilis, Dissoglottini mydrosoma*, wasps	wasp	(Torres et al. 2012)
SUBLANCEOLATA	*sublanceolata*	Mexico (Yucatan)	hummingbirds	bird	(Janzen 1968b), (Macdougal 2004)
TARMINIANA	*tarminiana*	Peru	*Ensifera ensifera*	bird	(Abrahamczyk et al. 2014)
TRIDACTYLITES	*tridactylites*	NA	moth? (abundant lepidoptera scales found inside flower)	moth	(Porter-Utley 2003)
TULAE	*tulae*	NA	hummingbird	bird	(Ulmer 2004)
UNIPETALA	*unipetala*	Bellavista Cloud Forest Reserve, Ecuador	*Anoura fisulata*	bat	(Jorgensen et al. 2012)
URNIFOLIA	*urnaefolia*	NA	*Xylocopa* (6 species)*, *Bombus tucumanus*, Centris spp, Erinnyis ello (Sphingidae),* hummingbirds (including *Chlorostilbon aureoventris*)	bee	(García and Hoc 2001)
VIRIDESCENS	viridescens	Ecuador	chewing pollinator? (no nectar, instead a fleshy corona offered; but not actual observations)	other	(Jorgensen et al. 2012)
VIRIDIFLORA	viridiflora	Coastal Oaxaca, Mexico	hummingbird	bird	(Macdougal 1992)
VITIFOLIA	vitifolia	Costa Rica	hummingbird: *Phaethornis superciliosus*	bird	(Skutch 1952)
VITIFOLIA	vitifolia	Costa Rica	hummingbirds: 4 unidentified spp.	bird	(Janzen 1968b)
VITIFOLIA	vitifolia	Costa Rica	hummingbird: *Phaethornis superciliosus*	bird	(Snow 1982)
WEBERBAUERI	weberbaueri	NA	bat	bat	(Abrahamczyk et al. 2014)

SI Table 4: Floral Scent Chemistry

- ID.Criteria: Method by which identity of chemical was established; MS = NIST mass spectra, K = Kovats Index on DB5 column, run against C7-C30 alkane mix
- **See attached Excel file 'SI_Table4_Floral_Scent_Chemistry**

SI Table 5: *Passiflora* species sampling for all plant-based analyses in this manuscript; morpho = floral morphology data set, chem = floral scent data set, obs. poll = pollinator observation records, phylo = gene sequence data; notations involving + are set intersections of the species in each of these data sets.

actinia, adenopoda, affinis, alata, allantophylla, amazonica, ambigua, amethystina, amoena, ampullacea, anastomosa ns, andina, antioquiensis, apetala, arborea, arida, aurantia, auriculata, bicornis, biflora, boenderi, bracteosa, caerulea, capsularis, cincinnata, cinnabarina, cirrhiflora, citrifolia, citrina, chrysophylla, coactilis, coccinea, colinvauxii, coriac ea, costaricensis, crispolanata, cumbalensis, cuneata, cupraea, deltoifolia, dioscoreifolia, discophora, edulis, exura, f oetida, galbana, garckei, gibertii, gilbertiana, gracilens, guatemalensis, haematostigma, hahnii, harlingii, helleri, herb ertiana, holosericea, incarnata, indecora, insignis, jamesonii, jorullensis, karwinskii, kawensis, kermesina, lanata, lan cetillensis, laurifolia, leptomischa, ligularis, lindeniana, lobata, loefgrenii, loxensis, luetzelburgii, luzmarina, macrop hylla, macropoda, maliformis, mandonii, manicata, mansoi, mathewsii, membranacea, menispermifolia, mexicana, microstipula, miersii, misera, mixta, mooreana, morifolia, mucronata, murucuja, obovata, oerstedii, organensis, ovali s, parritae, peduncularis, penduliflora, perfoliata, pilosicorona, pinnatistipula, pittieri, platyloba, punctata, quadrangu laris, quadriglandulosa, racemosa, recurva, reflexiflora, rhamnifolia, roseorum, rovirosae, rubra, sagasteguii, sanctae barbarae, sanguinolenta, seemannii, serratifolia, serratodigitata, setacea, sexflora, sexocellata, speciosa, sphaerocarp a, sprucei, standleyi, suberosa, sublanceolata, subpeltata, tarminiana, tatei, telesiphe, tenuifila, tina, trifasciata, trifoli ata, tripartita, trisecta, tucumanensis, tulae, umbilicata, unipetala, vespertilio, viridiflora, vitifolia, xiikzodz, yucatane nsis

DATA SET	N	ANALYSES	*PASSIFLORA* SPECIES INCLUDED	NOTES
MORPHO	150	morpho random forest test set	actinia, adenopoda, affinis, alata, allantophylla, am azonica, ambigua, amethystina, amoena, ampullace a, anastomosans, andina, antioquiensis, apetala, arb orea, arida, aurantia, auriculata, bicornis, biflora, bo enderi, bracteosa, caerulea, capsularis, cincinnata, c innabarina, cirrhiflora, citrifolia, citrina, chrysophyl la, coactilis, coccinea, colinvauxii, coriacea, costari censis, crispolanata, cumbalensis, cuneata, cupraea, deltoifolia, dioscoreifolia, discophora, edulis, exura , foetida, galbana, garckei, gibertii, gilbertiana, grac ilens, guatemalensis, haematostigma, hahnii, harlin gii, helleri, herbertiana, holosericea, incarnata, inde cora, insignis, jamesonii, jorullensis, karwinskii, ka wensis, kermesina, lanata, lancetillensis, laurifolia, leptomischa, ligularis, lindeniana, lobata, loefgrenii , loxensis, luetzelburgii, luzmarina, macrophylla, m acropoda, maliformis, mandonii, manicata, mansoi, mathewsii, membranacea, menispermifolia, mexica na, microstipula, miersii, misera, mixta, mooreana, morifolia, mucronata, murucuja, obovata, oerstedii, organensis, ovalis, parritae, peduncularis, pendulifl ora, perfoliata, pilosicorona, pinnatistipula, pittieri, platyloba, punctata, quadrangularis, quadriglandulo sa, racemosa, recurva, reflexiflora, rhamnifolia, ros eorum, rovirosae, rubra, sagasteguii, sanctaebarbara e, sanguinolenta, seemannii, serratifolia, serratodigi tata, setacea, sexflora, sexocellata, speciosa, sphaer ocarpa, sprucei, standleyi, suberosa, sublanceolata,	

			subpeltata, tarminiana, tatei, telesiphe, tenuifila, tina, trifasciata, trifoliata, tripartita, trisecta, tucumanensis, tulae, umbilicata, unipetala, vespertilio, viridiflora, vitifolia, xiikzodz, yucatanensis	
CHEM	70	chem ~ inferred poll ANOSIM; chem ~ inferred poll NMDS; scent emission level ANOVA and pairwise contrasts (see SI materials and methods)	actinia, ambigua, amethystina, antioquiensis, aurantia, biflora, boenderi, bogotensis, bryonioides, caerulea, cincinnata, citrina, coccinea, coriacea, deltoifolia, edulis, galbana, giberti, herbertiana, holosericea, incarnata, lancetillensis, laurifolia, ligularis, lindeniana, maliformis, manicata, manta, miersii, misera, mollissima, mooreana, morifolia, mucronata, murucuja, nephrodes, occidentalis.sp.nov.ined., oerstedii, penduliflora, perfoliata, platyloba, punctata, quadrangularis, quadriglandulosa, racemosa, rubra, rugosissima, sanguinolenta, serratifolia, serratodigitata, serrulata, sexflora, sexocellata, sprucei, standleyi, suberosa, sublanceolata, subpeltata, sunburst, tarminiana, tatei, telesiphe, aff.oerstedii, trifasciata, trisecta, tucumanensis, tulae, vitifolia, xdecaisneana, yucatanensis	
PHYLO	244	phylogeny (Bayesian)	actinia, adenopoda, adulterina, aff.ekmanii, aff.giberti, aff.oerstedii, aff.rugo, alata, allantophylla, alnifolia, altebilobata, amazonica, ambigua, amethystina, amoena, ampullacea, anadenia, antioquiensis, apetala, apoda, arbelaezii, arborea, arida, aurantia, aurantioides, auriculata, azulitensis.Molinari, berteroana, bicornis, bicrura, bicuspidata, biflora, boenderi, bogotensis, bracteosa, bryonioides, caerulea, calcicola, campanulata, capsularis, chelidonea, chrysosepala, cincinnata, cinnabarina, cirrhiflora, citrina, coactilis, cobanensis, coccinea, colimensis, colinvauxii, coriacea, costaricensis, cubensis, cumbalensis, cuneata, cupiformis, cupraea, deidamioides, deltoifolia, discophora, dolichocarpa, eberhardtii, edmundoi, edulis, eichleriana, elegans, escobariana, exsudans, exura, fimbriatistipula, foetida, gabrielliana, galbana, garckei, giberti, gilbertiana, gracilens, gracilis, gracillima, guatemalensis, haematostigma, hahnii, harlingii, helleri, henryi, herbertiana, hirtiflora, hollrungii, holosericea, ichthyura, incarnata, indecora, insignis, jatunsachensis, jilekii, jugorum, juliana, jussieui, karwinskii, kawensis, kermesina, kuranda, lanata, lancearia, lancetillensis, lancifolia, laurifolia, leptoclada, leptomischa, ligularis, lindeniana, lobata, lobbii.subsp.ayacuchoensis, loxensis, lutea, luzmarina, macrophylla, macropoda, maestrensis, maliformis, mandonii, manicata, mansoi, mathewsii, membranacea, mendoncaei, menispermifolia, mexicana, micropetala, microstipula, miersii, misera, mixta, mollissima, moluccana.var.teysmanniana, monadelpha, mooreana, morifolia, mucronata, multiflora, murucuja, nephrodes, nitida, oblongata, obovata, obtusifolia, occidentalis.sp.nov.ined, oerstedii, orbiculata, organensis, ornithoura, ovalis, pardifolia, parritae, pavonis, pedicellaris, peduncularis, pendens, penduliflora, per	238 Passiflora species (shown in this table), 244 species including outgroups

			akensis, perfoliata, pilosa, pilosicorona, pinnatistipula, pittieri, platyloba, pohlii, porphyretica, punctata, pusilla, pyrrhantha, quadrangularis, quadriglandulosa, quindiensis, racemosa, recurva, reflexiflora, riparia, rovirosae, rubra, rufa, rugosa, rugosissima, sagasteguii, sanctaebarbarae, sandrae, sanguinolenta, seemannii, serratifolia, serratodigitata, serrulata, setacea, setulosa, sexflora, sexocellata, siamica, sicyoides, sidifolia, sodiroi, solomonii, speciosa, sphaerocarpa, sprucei, standleyi, suberosa, subpeltata, tacanensis, tacsonioides, talamancensis, tarminiana, tatei, telesiphe, tenella, tenuifila, tenuiloba, tetrandra, tina, tricuspis, trifasciata, trifoliata, trisecta, truncata, tryphostemmatoides, tuberosa, tucumanensis, tulae, umbilicata, urnifolia, urubiciensis, vespertilio, villosa, vitifolia, weigendii, wilsonii, xiikzodz, yucatanensis, zamorana	
POLL	76		actinia, adenopoda, affinis, alata, alnifolia, amethystina, ampullacea, antioquiensis, apetala, arizonica, aurantia, bicornis, biflora, brevifila, caerulea, capsularis, chrysophylla, cincinnata, citrina, coccinea, colombiana, coriacea, dioscoreifolia, edulis, foetida, galbana, gilbertiana, hahnii, helleri, herbertiana, holosericea, incarnata, jamesonii, jorullensis, kermesina, lancetillensis, lobata, loefgrenii, longiracemosa, lutea, luzmarina, malacophylla, manicata, matthewsii, membranacea, mexicana, miersii, misera, mixta, mollissima, mooreana, mucronata, murucuja, obovata, ovalis, penduliflora, perfoliata, platyloba, pohlii, quadrangularis, quadriglandulosa, roseorum, sanguinolenta, sexflora, sexocellata, speciosa, suberosa, sublanceolata, tarminiana, tridactylites, trisecta, tulae, unipetala, urnifolia, viridiflora, vitifolia	
MORPHO + POLL	64	Morpho random forest training set (raw data)	actinia, adenopoda, affinis, alata, amethystina, ampullacea, antioquiensis, apetala, aurantia, bicornis, biflora, caerulea, capsularis, chrysophylla, cincinnata, citrina, coccinea, coriacea, dioscoreifolia, edulis, foetida, galbana, gilbertiana, hahnii, helleri, herbertiana, holosericea, incarnata, jamesonii, jorullensis, kermesina, lancetillensis, lobata, loefgrenii, luzmarina, manicata, membranacea, mexicana, miersii, misera, mixta, mooreana, mucronata, murucuja, obovata, ovalis, penduliflora, perfoliata, platyloba, quadrangularis, quadriglandulosa, roseorum, sanguinolenta, sexflora, sexocellata, speciosa, suberosa, sublanceolata, tarminiana, trisecta, tulae, unipetala, viridiflora, vitifolia	
MORPHO + POLL (EXCLUDING MOTH-POLLINATED SPP.)	62	Morpho random forest training set (balanced samples)	actinia, adenopoda, affinis, alata, amethystina, ampullacea, antioquiensis, apetala, aurantia, bicornis, biflora, caerulea, capsularis, chrysophylla, cincinnata, citrina, coccinea, coriacea, dioscoreifolia, edulis, foetida, galbana, gilbertiana, helleri, herbertiana, holosericea, incarnata, jamesonii, jorullensis, kermesina, lancetillensis, lobata, loefgrenii, luzmarina, manic	Moth-pollinated species removed to facilitate down-sampling

			ata, membranacea, mexicana, miersii, misera, mixta , mucronata, murucuja, obovata, ovalis, penduliflor a, perfoliata, platyloba, quadrangularis, quadrigland ulosa, roseorum, sanguinolenta, sexflora, sexocellat a, speciosa, suberosa, sublanceolata, tarminiana, tri secta, tulae, unipetala, viridiflora, vitifolia	
PHYLO + MORPHO	132	Ancestral state reconstruction (pollinator predicted as in morpho random forest test set); Mantel for phylogenetic signal in floral morphology	actinia, adenopoda, alata, allantophylla, amazonica, ambigua, amethystina, amoena, ampullacea, antioq uiensis, apetala, arborea, arida, aurantia, auriculata, bicornis, biflora, boenderi, bracteosa, caerulea, caps ularis, cincinnata, cinnabarina, cirrhiflora, citrina, c oactilis, coccinea, colinvauxii, coriacea, costaricens is, cumbalensis, cuneata, cupraea, deltoifolia, disco phora, edulis, exura, foetida, galbana, garckei, gilbe rtiana, gracilens, guatemalensis, haematostigma, ha hnii, harlingii, helleri, herbertiana, holosericea, inca rnata, indecora, insignis, karwinskii, kawensis, ker mesina, lanata, lancetillensis, laurifolia, leptomisch a, ligularis, lindeniana, lobata, loxensis, luzmarina, macrophylla, macropoda, maliformis, mandonii, m anicata, mansoi, mathewsii, membranacea, menispe rmifolia, mexicana, microstipula, miersii, misera, m ixta, mooreana, morifolia, mucronata, murucuja, ob ovata, oerstedii, organensis, ovalis, parritae, pedunc ularis, penduliflora, perfoliata, pilosicorona, pinnati stipula, pittieri, platyloba, punctata, quadrangularis, quadriglandulosa, racemosa, recurva, reflexiflora, r ovirosae, rubra, sagasteguii, sanctaebarbarae, sangu inolenta, seemannii, serratifolia, serratodigitata, set acea, sexflora, sexocellata, speciosa, sphaerocarpa, sprucei, standleyi, suberosa, subpeltata, tarminiana, tatei, telesiphe, tenuifila, tina, trifasciata, trifoliata, t risecta, tucumanensis, tulae, umbilicata, vespertilio, vitifolia, xiikzodz, yucatanensis	
PHYLO + POLL	58	Ancestral state reconstruction (observed pollinator only)	actinia, adenopoda, alata, alnifolia, amethystina, am pullacea, antioquiensis, apetala, aurantia, bicornis, biflora, caerulea, capsularis, cincinnata, citrina, coc cinea, coriacea, edulis, foetida, galbana, gilbertiana, hahnii, helleri, herbertiana, holosericea, incarnata, k ermesina, lancetillensis, lobata, lutea, luzmarina, m anicata, membranacea, mexicana, miersii, misera, mixta, mooreana, mucronata, murucuja, obovata, o valis, penduliflora, perfoliata, platyloba, pohlii, qua drangularis, quadriglandulosa, sanguinolenta, sexfl ora, sexocellata, speciosa, suberosa, tarminiana, tris ecta, tulae, urnifolia, vitifolia	Excl. *P. mollissim a*
CHEM + POLL	38	Chem ~ poll ANOSIM, chem ~ poll NMDS	actinia, amethystina, antioquiensis, aurantia, biflora , caerulea, cincinnata, citrina, coccinea, coriacea, ed ulis, galbana, herbertiana, holosericea, incarnata, la ncetillensis, manicata, miersii, misera, mollissima, mooreana, mucronata, murucuja, penduliflora, perf oliata, platyloba, quadrangularis, quadriglandulosa, sanguinolenta, sexflora, sexocellata, suberosa, subl anceolata, tarminiana, trisecta, tulae, vitifolia	incl. *P. 'Sunburst'* as *P. jorullensis* ; see si materials and methods)

CHEM + PHYLO	61	Mantel chem ~ inferred poll (pollinator predicted as in morpho random forest test set + 12 inferred by authors based on floral morphology); Mantel ~ chem ~ inferred poll + phylo	actinia, ambigua, amethystina, antioquiensis, aurantia, biflora, boenderi, bogotensis, bryonioides, caerulea, cincinnata, citrina, coccinea, coriacea, deltoifolia, edulis, giberti, herbertiana, holosericea, incarnata, lancetillensis, laurifolia, ligularis, maliformis, manicata, miersii, misera, mollissima, mooreana, morifolia, mucronata, murucuja, nephrodes, oerstedii, penduliflora, perfoliata, platyloba, punctata, quadrangularis, quadriglandulosa, racemosa, rubra, sanguinolenta, serratifolia, serratodigitata, serrulata, sexflora, sexocellata, sprucei, standleyi, suberosa, subpeltata, tarminiana, tatei, telesiphe, trifasciata, trisecta, tucumanensis, tulae, vitifolia, yucatanensis	Excl. P. aff. oerstedii, P. rugosissima, P. lindeniana, P. galbana
CHEM + PHYLO	65	PGLS scent emission rate, pairwise contrasts with phylANOVA	actinia, ambigua, amethystina, antioquiensis, aurantia, biflora, boenderi, bogotensis, bryonioides, caerulea, cincinnata, citrina, coccinea, coriacea, deltoifolia, edulis, galbana, giberti, herbertiana, holosericea, incarnata, lancetillensis, laurifolia, ligularis, lindeniana, maliformis, manicata, miersii, misera, mollissima, mooreana, morifolia, mucronata, murucuja, nephrodes, oerstedii, penduliflora, perfoliata, platyloba, punctata, quadrangularis, quadriglandulosa, racemosa, rubra, rugosissima, sanguinolenta, serratifolia, serratodigitata, serrulata, sexflora, sexocellata, sprucei, standleyi, suberosa, subpeltata, tarminiana, tatei, telesiphe, aff.oerstedii, trifasciata, trisecta, tucumanensis, tulae, vitifolia, yucatanensis	
MORPHO + CHEM	58	Morpho + chem random forest test set	actinia, ambigua, amethystina, antioquiensis, aurantia, biflora, boenderi, caerulea, cincinnata, citrina, coccinea, coriacea, deltoifolia, edulis, galbana, herbertiana, holosericea, incarnata, lancetillensis, laurifolia, ligularis, lindeniana, maliformis, manicata, miersii, misera, mooreana, morifolia, mucronata, murucuja, oerstedii, penduliflora, perfoliata, platyloba, punctata, quadrangularis, quadriglandulosa, racemosa, rubra, sanguinolenta, serratifolia, serratodigitata, sexflora, sexocellata, sprucei, standleyi, suberosa, sub lanceolata, subpeltata, tarminiana, tatei, telesiphe, trifasciata, trisecta, tucumanensis, tulae, vitifolia, yucatanensis	
MORPHO + CHEM + PHYLO	57	Comparable Mantel tests for phylogenetic signal in and correlation between morphological and chemical data sets, (morpho ~ phylo, chem ~ phylo, morpho ~ chem, morpho ~ phylo + chem, morpho ~ inferred poll + chem)	actinia, ambigua, amethystina, antioquiensis, aurantia, biflora, boenderi, caerulea, cincinnata, citrina, coccinea, coriacea, deltoifolia, edulis, galbana, herbertiana, holosericea, incarnata, lancetillensis, laurifolia, ligularis, lindeniana, maliformis, manicata, miersii, misera, mooreana, morifolia, mucronata, murucuja, oerstedii, penduliflora, perfoliata, platyloba, punctata, quadrangularis, quadriglandulosa, racemosa, rubra, sanguinolenta, serratifolia, serratodigitata, sexflora, sexocellata, sprucei, standleyi, suberosa, subpeltata, tarminiana, tatei, telesiphe, trifasciata, trisecta, tucumanensis, tulae, vitifolia, yucatanensis	

MORPHO + CHEM + POLL	36	Morpho + chem random forest training set	actinia, amethystina, antioquiensis, aurantia, biflora, caerulea, cincinnata, citrina, coccinea, coriacea, edulis, galbana, herbertiana, holosericea, incarnata, lancetillensis, manicata, miersii, misera, mooreana, mucronata, murucuja, penduliflora, perfoliata, platyloba, quadrangularis, quadriglandulosa, sanguinolenta, sexflora, sexocellata, suberosa, sublanceolata, tarminiana, trisecta, tulae, vitifolia
MORPHO + CHEM + POLL + PHYLO	35	Mantel morpho ~ obs poll, morpho ~ obs poll + phylo	actinia, amethystina, antioquiensis, aurantia, biflora, caerulea, cincinnata, citrina, coccinea, coriacea, edulis, galbana, herbertiana, holosericea, incarnata, lancetillensis, manicata, miersii, misera, mooreana, mucronata, murucuja, penduliflora, perfoliata, platyloba, quadrangularis, quadriglandulosa, sanguinolenta, sexflora, sexocellata, suberosa, tarminiana, trisecta, tulae, vitifolia

SUPPLEMENTAL INFORMATION – FIGURE LEGENDS

SI Figure 1: Full phylogeny of over 250 species of *Passiflora* with node posterior probabilities

A tree of over 250 species of *Passiflora*, created using both nuclear and chloroplast sequences from study plants and from sequences available on Genbank, confirmed subgenera (*Astrophea, Deidamioides, Decaloba, Passiflora, Tetrapathea*) put forth in previous studies. The morphological data set spans the four largest subgenera, with representation from all but small subgenus *Tetrapathea*. The chemical data set primarily represents the two largest subgenera, the *Decaloba* and the *Passiflora*, but has a sample from the basal-most subgenus in the *Passiflora*, the *Astrophea*. The pollination data set has observations primarily from the largest two subgenera, likely because they represent such a large proportion of the species in the genus, but also from small subgenus *Deidamioides,* which includes bat-pollinated species.

SI Figure 2: Ancestral state reconstruction, with pollinator inferred by morphology

Random forest based on quantitative morphology was used to infer the major pollinator of many species for which we did not have direct pollinator observations. The analysis confirmed bee as the ancestral pollinator of the genus and clarified and increased the number of likely transitions from pollination by one pollinator type to another.

SI Figure 3: Chemical clustering by subgenus, illustrating phylogenetic signal

(A-C) Chemical data showed phylogenetic signal, but chemical clusters by subgenus were distinct from those by pollinator: An NMDS on chemical abundance (**A**, Bray-Curtis) shows clustering and separation by subgenus, but clusters are orthogonal from pollinator type clusters. Chemical identity (**B**, Jaccard), and chemical relative ratios (**C,** Bray-Curtis) showed subgenus

clusters that were barely distinguishable from one another. For all NMDS, stress > 0.2 and < 0.25.

SI Figure 4: Antennal responses to individual chemical constituents of floral scent (SI)

(**A**) PCA of antennal responses to *P. quadrangularis* odors (GC-EAD) comparing *M. sexta* moths to *B. impatiens* and *X. californica* bees.

(**B-E**) Example traces of insect antennae responding to the chemical constituents of bee-pollinated *P. quadrangularis* as they elute from the GCMS over time (GC-EAD). Panels (B-D) show the antennae responding to a subset of chemicals, and with different intensities of response for *M. sexta, B. impatiens,* and *X. californica* respectively. Panel E shows the chromatogram of chemicals making up the floral scent. Each peak denotes an individual chemical, and the peak size denotes the abundance of that chemical in the floral scent.

SI Figure 5: Bees and moths respond to individual odorants differently (SI)

(**A**) Differences in responses to tested chemicals of normalized concentration in the air between *B. impatiens* and *M. sexta*. Error bars denote standard error and bars in bar chart show mean antennal response to each chemical, normalized the maximum response per antennal preparation.

(**B**) Clustering of *Manduca* and *Bombus* responses to odorants from a variety of chemical classes show significant separation with k-means clustering, suggesting variation between these species were more different than variation in responses within species.

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SUPPLEMENTAL INFORMATION – STATISTICAL TABLES DOCUMENT t1-t37

SI doc t1: PERMANOVA, multivariate morphology often explained by observed pollinator; n = 64, for breakdown by observed pollinator type, see table 2 in this document.

	R^2	**Bonferroni-adjusted p-value**
overall	0.205	< 0.008**
bee-bird	0.352	< 0.008**
bee-wasp	0.166	0.016*
bee-bat	0.211	< 0.008**
bee-moth	0.029	1.000
bee- multiple (BBB)	0.216	< .008**
bird-bat	0.1491	0.032*
bird-multiple (BBB)	0.1476	0.096

SI doc t2: Sample size by observed pollinator type for morphological data

pollinator	**n**
overall	64
bat	6
bee	24
bird	24
moth	2
wasp	8

SI doc t3: Mantel tests show significant relationship between morphology and observed pollinator both with and without taking phylogenetic relatedness into account (n = 35) using a sample consisting of the species that occurred in all data sets in the study (floral scent, floral morphology, observed pollinator, phylogeny)

	n	**R**	**p**

Morpho ~ obs. poll.	35	0.17	0.002**
Morpho ~ phylo. + obs. poll.	35	0.16	0.005**

SI doc t4: Full raw morphology random forest confusion matrix; OOB estimate of error: 20.31%; overall model error same. First column is actual pollinator, top row is pollinator predicted by model; n = 64, sampling as in table 2 in this document.

	bat	**bee**	**bird**	**moth**	**wasp**	**class error**
bat	2	2	2	0	0	0.667
bee	0	22	0	0	2	0.083
bird	1	0	23	0	0	0.042
moth	0	2	0	0	0	1.000
wasp	0	4	0	0	4	0.500

SI doc t5: Morphology excluding sex organ random forest confusion matrix; OOB estimate of error: 29.69%, overall model error same. First column is actual pollinator, top row is pollinator predicted by model; n = 64, sampling as in table 2 in this document.

	bat	**bee**	**bird**	**moth**	**wasp**	**class error**
bat	2	2	2	0	0	0.667
bee	1	18	3	0	2	0.250
bird	0	3	21	0	0	0.125
moth	0	2	0	0	0	1.000
wasp	0	4	0	0	4	0.500

SI doc t6: Random forest confusion matrix for reduced morphological data set (morpho-chem data set overlap, n = 35); OOB estimate of error rate: 20%, overall model error same. First column is actual pollinator, top row is pollinator predicted by model.

	bat	**bee**	**bird**	**moth**	**wasp**	**class error**
bat	1	1	1	0	0	0.667

bee	0	12	0	0	2	0.143
bird	1	0	13	0	0	0.071
moth	0	1	0	0	0	1.000
wasp	0	1	0	0	2	0.333

SI doc t7: Full raw morphology random forest with balanced sampling summary of confusion with and without sex organ included in the analysis; sampling: n = 62 (excluding n = 2 moth-pollinated species because of low sample size), per run sampling: n(wasp) =6, n(bat) = 6, n(bee) = 8, n(bird) = 8; parameters: variables tried per node = 10, number of decision trees = 2000

	n	**class error (all traits)**	**class error (excl. sex organ)**
Overall / OOB	62	0.145	0.229
bat	6	0.167	0.5
bee	24	0.208	0.25
bird	24	0.083	0.167
wasp	8	0.125	0.125

SI doc t8: Balanced random forest confusion matrix for reduced morphological data set (morpho-chem data set overlap, n = 35); OOB estimate of error rate: 22.58%

	n	**class error**
Overall / OOB	35	0.229
bat	4	0.250
bee	14	0.143
bird	14	0.071
wasp	3	0.333

SI doc t9: Cross validation of full morphology balanced RF shows that sex organ and petal color classify flowers into pollination groups about as well as models using additional morphological traits.

Num. Vars. in Model	14	10	7	5	3	2	1
Error Rate	0.161	0.161	0.161	0.177	0.209	0.177	0.270

SI doc t10: Cross validation of morphology balanced RF excluding sex organ as a trait

Num. Vars. in Model	13	9	6	4	3	2	1
Error Rate	0.226	0.306	0.274	0.323	0.419	0.565	0.532

SI doc t11: ANOSIM on chemical abundance ~ observed pollinator (NMDS stress = 0.227), n = 38, n_{bee} = 14, n_{bird} = 15, n_{wasp} = 4 , n_{bat} = 4, n_{moth} = 1

	statistic	**p-value**	**BH-adjusted p-value**
overall	0.196	0.001	0.007 **
bee-bird	0.141	0.009	0.021*
bee-wasp	0.164	0.185	0.022*
bee-bat	0.399	0.200	0.035*
bird-wasp	0.225	0.080	0.112
bird-bat	0.410	0.004	0.014*
wasp-bat	0.073	0.332	0.332

SI doc t12: ANOSIM on chemical abundance ~ inferred pollinator (NMDS stress = 0.228); n = 70, n_{bee} = 38, n_{bird} = 16, n_{wasp} = 10 , n_{bat} = 5, n_{moth} = 1

	statistic	**p-value**	**BH-adjusted p-value**
overall	0.411	0.001	0.0035**
bee-bird	0.325	0.001	0.0035**
bee-wasp	0.464	0.002	0.0042**

bee-bat	0.468	0.003	0.0042**
bird-wasp	0.249	0.003	0.0042**
bird-bat	0.315	0.017	0.0198*
wasp-bat	0.187	0.073	0.0730

SI doc t13: ANOSIM on presence/absence chemical data ~ inferred pollinator (NMDS stress = 0.264), n = 70, n_{bee} = 38, n_{bird} = 16, n_{wasp} = 10 , n_{bat} = 5, n_{moth} = 1

	statistic	**p-value**	**BH-adjusted p-value**
overall	0.333	0.001	0.0023**
bee-bird	0.286	0.001	0.0023**
bee-wasp	0.437	0.001	0.0023**
bee-bat	0.422	0.002	0.0035**
bird-wasp	0.184	0.019	0.0210*
bird-bat	0.422	0.003	0.0042**
wasp-bat	0.340	0.021	0.0210*

SI doc t14: ANOSIM on relative individual chemical data ~ inferred pollinator (NMDS stress = 0.228); n = 70, n_{bee} = 38, n_{bird} = 16, n_{wasp} = 10 , n_{bat} = 5, n_{moth} = 1

	statistic	**p-value**	**BH-adjusted p-value**
overall	0.372	0.001	0.0023**
bee-bird	0.327	0.001	0.0023**
bee-wasp	0.463	0.001	0.0023**
bee-bat	0.426	0.003	0.0042**
bird-wasp	0.273	0.003	0.0042**
bird-bat	0.253	0.025	0.0291*
wasp-bat	-0.031	0.571	0.5710

SI doc t15: Mean floral scent emission rates were significantly explained by inferred pollinator; type III ANOVA for unbalanced samples, n = 70, n_{bee} = 38, n_{bird} = 16, n_{wasp} = 10 , n_{bat} = 5, n_{moth} = 1; $p = 3.985 \times 10^{-5}$

	Mean Emission (log)	**SE**	**Df**	**95% CI (Sidak adjusted for 4 estimates)**	**Group**
Bat	9.26	0.437	65	(8.141, 10.380)	A
Bee	8.61	0.159	65	(8.202, 9.013)	A
Bird	7.47	0.244	65	(6.847, 8.099)	B
wasp	7.42	0.309	65	(6.647, 8.230)	B

SI doc t16: Pairwise contrasts for scent emission differences by pollinator demonstrated that bat- and bee- pollinated Passiflora species had significantly higher levels of scent emission than wasp- and bird- pollinated species, Tukey-Kramer post hoc test for unbalanced samples; n_{bee} = 38, n_{bird} = 16, n_{wasp} = 10 , n_{bat} = 5, n_{moth} = 1

Contrast	**Estimate**	**SE**	**df**	**t-ratio**	**p-value**
bat - bee	0.653	0.465	65	1.404	0.501
bat - bird	1.822	0.535	65	3.570	0.004**
bat - wasp	1.822	0.535	65	3.404	0.006**
bee - bird	1.135	0.291	65	3.896	0.001**
bee - wasp	1.169	0.347	65	3.366	0.007**
bird - wasp	0.034	0.394	65	0.087	0.999

SI doc t17: PGLS scent emission rate ~ inferred pollinator (phylogenetic generalized least squares of $\log_{10}$(24 hr emission) against pollinator inferred by morphology), n = 65

	$\log_{10}$(24-hour emission) estimates
bat	8.959
bee	8.121
bird	7.021
wasp	8.319

SI doc t18: Pairwise contrasts for scent emission rate (log-transformed) with phylogenetic correction (phylogenetic ANOVA) demonstrated that bird-pollinated species emitted significantly less than their ancestors, while bat-pollinated species tended to emit more, n = 65

contrasts	**BH corrected p-values**
overall	0.045 *
bat-bird	0.006 ***
bee-bird	0.018 *
bat-bee	0.113
bat-wasp	0.113
bee-wasp	0.488
bird-wasp	0.844

SI doc t19: The absolute number of chemicals making up a floral scent was significantly explained by inferred pollinator type (Type III ANOVA for unbalanced samples, p = 0.002, n = 70)

	Mean Num. Chems	**SE**	**df**	**95% CI (Sidak adjusted for 4 estimates)**	**Group**
bat	25.800	9.890	65	(6.048, 45.552)	A, B
bee	36.395	3.587	65	(29.230, 43.559)	A
bird	12.125	5.529	65	(1.083, 23.167)	B
wasp	15.200	6.993	65	(1.233, 29.166)	B

SI doc t20: Pairwise contrasts did not show significant differences between pollinator types in the number of chemicals making up floral scent, Tukey-Kramer post hoc tests for unbalanced samples (n = 70)

Contrast	Estimate	SE	df	t-ratio	p-value
bat-bee	-10.595	10.521	65	-1.007	0.7459
bat-bird	13.675	11.330	65	1.207	0.6247
bat-wasp	10.600	12.113	65	0.875	0.8177
bee-bird	24.269	6.591	65	3.682	0.0026 *
bee-wasp	21.195	7.859	65	2.697	0.0431 *
bird-wasp	-3.075	8.915	65	-0.345	0.9858

SI doc t21: Phylogenetically corrected (PGLS) estimates for mean number of chemicals emitted by Passiflora species by inferred pollinator; estimates were not significantly different (phylogenetic ANOVA p = 0.178, n = 65)

	Mean. Num. Chems.
bat	35.0
bee	31.4
bird	13.4
wasp	27.8

SI doc t22: Pairwise contrasts for number of distinct chemicals making up a floral scent with phylogenetic correction using phylogenetic ANOVA demonstrated that bird-pollinated species

emitted significantly fewer chemicals than their bee-pollinated ancestors; no other contrasts showed significant differences in number of chemicals emitted, n = 65.

contrast	**BH-corrected p -values**
overall	0.176
bee-bird	0.018**
bat-bird	0.404
bat-bee	0.404
bee-wasp	0.761
bat-wasp	0.812
bird-wasp	0.812

SI doc t23: Bee- and bird- pollinated Passiflora species were the only types that were reliably discriminated from one another using RF with chemical data against observed pollinator using a variety of parameters, sampling schemes, and types of chemical data sets (raw, percentage, presence/absence); including balanced (8 bee-, bird- and 4 bat-, wasp- pollinated species per run, constrained by the small sample size of bat-pollinated species) versus raw, 20000 decision trees and 500 variables tried per split versus 2000 decision trees and 22 variables tried per split, raw versus relative versus presence/absence chemical data sets; n = 38 in training set and n = 72 in test set.

	mean class error	**class error range**
bee	0.214	[0.100, 0.357]
bird	0.203	[0.133, 0.430]

SI doc t24: Full raw individual chemical ~ inferred pollinator random forest confusion matrix; OOB estimate of error: 20.31%, , overall model error: 37.14% (2000 trees, 22 variables tried at each split); n = 70

	bat	**bee**	**bird**	**moth**	**wasp**	**class error**
bat	0	3	2	0	0	1.000
bee	0	33	4	0	1	0.132

bird	0	5	10	0	1	0.375
moth	0	1	0	0	0	1.000
wasp	0	5	4	0	1	0.900

SI doc t25: Full binary individual chemical ~ inferred pollinator random forest confusion matrix; OOB estimate of error: 35.71%, overall model error same (2000 trees, 22 variables tried at each split); n = 70.

	bat	**bee**	**bird**	**moth**	**wasp**	**class error**
bat	0	3	2	0	0	1.000
bee	0	34	4	0	0	0.105
bird	0	5	11	0	0	0.313
moth	0	1	0	0	0	1.000
wasp	0	5	5	0	0	1.000

SI doc t26: Full percentage individual chemical ~ inferred pollinator random forest confusion matrix; OOB estimate of error: 40%, overall model error same (2000 trees, 22 variables tried at each split); n = 70

	bat	bee	bird	moth	Wasp	class error
bat	0	5	0	0	0	1.000
bee	0	35	2	0	1	0.008
bird	0	10	6	0	0	0.625
moth	0	1	0	0	0	1.000
wasp	0	9	0	0	1	0.900

SI doc t27: Many chemicals were unique to bat-pollinated species of Passiflora. Notable chemicals that were unique to bat-pollinated species included several lactones, an oxime, and oxygenated alkanes. Most had odors described as “fermented,” “fatty” or like chicken fat, “creamy”, or “fruity” to the human nose (Goodrich et al. 2006; Company 2015). The terpenes that were unique to bat-pollinated species were all described as “woody,” (Company 2015) in

contrast to the “sweet” or “fresh” terpenes that were more common in bee flowers ((Company 2015), as in (Harborne 2001; Goodrich and Raguso 2009)).

Class	**Chemical**	**Description (to the human nose)**
Lactone	2-(3H)-furanone, 5-heptyldihydro	creamy, waxy, green
	6-dodecenyl-gamma-lactone (dairy lactone)	sweet, dairy, creamy, fruity
	coumarin	sweet, new hay with bitter almond
Oxime	butanal, oxime	fermented
Alcohol	2,4-octadien-1-ol	fatty, chicken fat
	3-octen-1-ol	powerful, fruity, fatty, melon
Aldehyde	2,4-decadienal	oily, fatty, chicken fat, citrus
	tridecanal	aldehydic, soapy, citrus, fatty
	decen-4-al	aldehydic, citrus, fatty
Ester	nonanoic acid, methyl ester	fruity, waxy
	decanoic acid, methyl ester	fermented
Sesquiterpene	trans-isolongifolene	woody
	cycloisolongifolene	
monoterpene alcohol	2,6-octadiene-1,8-diol, 2,6-dimethyl	
	p-menth-8(10)-en-9-ol, cis (beta-terpinol)	woody
oxygenated aromatic	4,3-hydroxy-1-propenyl phenol (p-coumaryl alcohol)	(precursor to lactone coumarin)
	1,2-methoxy-4-(2-propenyl) benzene (estragole)	sweet, anise, spicy

SI doc t28: Chemicals unique to wasp-pollinated Passiflora. The set of 17 chemicals unique to wasp-pollinated species was dominated by alkanes on which there was no organoleptic

information available (2-Ethyl-4,6-dimethyltetrahydropyran; 5-tridecene; 6,9-heptadecadiene; bicyclo[2.2.2]octane, 2-methyl; bicyclo[5.3.0] decane; decane, 2,3,8-trimethyl; 3,4-octadiene, 7-methyl). Interestingly, some of these alkanes were structurally similar to common terpenes, suggesting that perhaps they were derived from these more common plant volatiles in the transition from bee- to wasp- pollination. Compounds of note for which organoleptic information was available included a lactone, two fruity-smelling aldehydes (Company 2015), and a strong fecal indole relative (Schiestl and Dötterl 2012).

Class	**Chemical**	**Description (to the human nose)**
lactone	2H-pyran-2-one, 6-hexyl tetrahydro (delta-undecalactone)	creamy, fatty, coconut, fruity, peach, waxy
indole-like	indole, 3-methyl (skatole)	very strong animal, fecal, indole, civet
aldehyde	2 pentenal, 2-methyl	fruity, aldehydic
	2-undecanal	fruity, orange peel, fresh
fatty acid	9,12,15-octadecatrienoic acid	faint fatty

SI doc t29: Bombus Passiflora EAG responses by pollinator, $n_{Bombus} = 9$; GLMM after taking individual into account shows that antennal responses by Bombus to the scent of bee-pollinated Passiflora species is significantly higher than to Passiflora species pollinated by other functional groups.

	Estimate	**Std. Error**	**p-value**	**95% CI**
Intercept (negative control)	2.22	0.58	0.00014***	(1.05, 3.39)
bat rel. to neg.control	2.79	1.07	0.00899***	(0.69, 4.89)
bee rel. to neg.control	3.38	0.51	2.619×10^{-11}***	(2.39, 4.38)
bird rel. to neg.control	2.52	0.57	9.252×10^{-6}***	(1.41, 3.64)
moth rel. to neg.control	2.39	1.06	0.0248*	(0.29, 4.50)
wasp rel. to neg. control	0.84	0.82	0.301	(-0.76, 2.46)
vegetative rel. to neg. control	0.44	0.60	0.466	(-0.74, 1.62)
positive control rel. to neg.control	20.71	0.73	0***	(19.28, 22.14)

SI doc t30: Manduca Passiflora EAG responses by pollinator; GLMM after taking individual into account, $n_{Manduca} = 8$

	Estimate	**Std. Error**	**p-value**	**95% CI**
Intercept (negative control)	1.56	0.49	1.647×10^{-3} ***	(0.57, 2.53)
bat rel. to neg.control	1.26	0.84	0.136	(-0.41, 2.92)
bee rel. to neg.control	2.57	0.50	2.996×10^{-7} ***	(1.58, 3.56)
bird rel. to neg.control	1.97	0.56	3.880×10^{-4}***	(0.88, 3.06)
moth rel. to neg.control	2.24	0.77	3.412×10^{-3}***	(0.73, 3.75)
wasp rel. to neg. control	1.16	0.68	0.0879	(-0.18, 2.49)
vegetative rel. to neg. control	0.90	0.59	0.124	(-0.25, 2.05)
positive control rel. to neg.control	12.84	0.75	0.000000 ***	(11.36, 14.31)

SI doc t31: Heliconius Passiflora EAG responses by pollinator; GLMM after taking individual into account, $n_{Heliconius} = 5$

	Estimate	**Std. Error**	**p-value**	**95% CI**
Intercept (negative control)	2.01	0.45	8.19×10^{-6}	(1.03, 2.99)
bat rel. to neg.control	0.24	0.75	0.75	(-1.24, 1.72)
bee rel. to neg.control	0.97	0.33	0.003	(0.32, 1.63)
bird rel. to neg.control	0.67	0.37	0.073	(-0.066, 1.41)
moth rel. to neg.control	1.29	0.82	0.12	(-0.34, 2.93)
wasp rel. to neg. control	1.69	0.49	5.41×10^{-4}	(0.73, 2.67)
vegetative rel. to neg. control	1.05	0.37	0.004	(0.33, 1.77)
positive control rel. to neg.control	4.96	0.49	0	(3.99, 5.93)

SI doc t32: GC-EADs on model pollinators with P. quadrangularis. Percentage of chemicals responded to by * that were also responded to by +

	Bombus *	Xylocopa *	Manduca *
Bombus +	/	92	92.3
Xylocopa +	100	/	100
Manduca +	52.2	52	/

SI doc t33: GC-EADs on model pollinators with P. mooreana. Percentage of chemicals responded to by * that were also responded to by +

	Bombus *	Manduca *
Bombus +	/	41.7
Manduca +	73.7	/

SI doc t34: ANOSIM on bee GC-EAG-filtered individual chemical data ~ inferred pollinator (NMDS stress = 0.197)

	statistic	p-value	BH-adjusted p-value
overall	0.420	0.001	0.0018**
bee-bird	0.411	0.001	0.0018**
bee-wasp	0.441	0.001	0.0018**
bee-bat	0.459	0.002	0.0028**
bird-wasp	0.065	0.175	0.1750
bird-bat	0.446	0.001	0.0018**
wasp-bat	0.272	0.049	0.0572

SI doc t35: ANOSIM on bee single-odorant EAG-filtered individual chemical data ~ inferred pollinator (NMDS stress = 0.194)

	statistic	p-value	BH-adjusted p-value
overall	0.427	0.001	0.0023**
bee-bird	0.404	0.001	0.0023**
bee-wasp	0.466	0.001	0.0023**
bee-bat	0.466	0.002	0.0028**
bird-wasp	0.069	0.162	0.1620
bird-bat	0.387	0.002	0.0028**
wasp-bat	0.276	0.039	0.0455*

SI doc t36: Cross validation of random forest using floral morphology and chemistry data together shows that adding chemical data did not improve the error rate above models using morphological data alone when using a small number of variables per split; there are 14 morphological variables, which all were of higher importance than chemical variables in the model. RF parameters: 2000 decision trees, 22 variables tried at each split

Num. Vars in Model	522	365	256	179	125	88	61	43	30	21	15	10	7	5	4	2	1
Error Rate	0.20	0.23	0.20	0.20	0.20	0.17	0.17	0.17	0.17	0.17	0.20	0.17	0.20	0.26	0.22	0.23	0.34

SI doc t37: Mantel tests for correlation between floral morphology, floral scent chemistry, and phylogenetic relatedness using the n = 57 species common to all three data sets show significant phylogenetic signal in both floral morphology and floral chemistry (also in the full data sets for each, in grey below), but also correlation between morphology and chemistry independent of relatedness, suggestion tandem evolution of these traits. When inferred pollinator is taken into account in the model, the correlation between the morphology and chemistry is eliminated.

	Mantel R	p-value	95% CI
Morpho ~ phylo	0.257 (0.156, n = 132)	0.001*** (0.001***)	(0.21, 0.30) ((0.13, 0.19))
Chem ~ phylo	0.131 (0.151, n = 65)	0.001*** (0.001***)	(0.981, 0.177) ((0.12, 0.19))
Morpho~ chem	0.129	0.002***	(0.090, 0.202)
Morpho ~ phylo + chem	0.101	0.008***	(0.051, 0.164)
Morpho ~ inf. poll + chem	0.032	0.24	no significant effect

SI STATISTICAL TABLES DOCUMENT - REFERENCES

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Chapter 3. HERBIVORY TRANSITIONS AND PLANT VOLATILE EVOLUTION IN THE PASSIFLORA-HELICONIINE SYSTEM

Herbivory transitions and plant volatile evolution in the *Passiflora-Heliconius* system

MR Clifford[1], N Cutlip[1], JH Chau[1], A Lance[1], J Eom[1], N Wornian[1], K Lungberg[1], A Stone[1], E Kim[1], and JA Riffell[1]

[1]University of Washington, Department of Biology, Seattle, WA 90095 USA;

INTRODUCTION

Insect herbivory plays a major role in shaping both man-made and natural ecosystems. In agricultural systems, insect herbivores and plant pathogens are responsible for the loss of an estimated 20-50% of all food grown worldwide (Maxmen 2013). In terrestrial systems, biodiversity is dominated by plants and their herbivores (Futuyma and Agrawal 2009). Because of the ubiquity of this interaction in the natural world, insect herbivores are a critical channel for energy flow to higher trophic levels and mediate a variety of ecologically important processes: insect herbivory contributes to the maintenance of community-level biodiversity (Brown 1994; De Deyn et al. 2003), habitat specialization, and the delimitation of plant distributions across both temperate and tropical ecosystems (Louda and Rodman 1996; Fine et al. 2004). Insect herbivory has also played a critical role in plant evolution. Studies which experimentally manipulated insect herbivory have demonstrated real-time evolutionary changes reflecting the trade-off between competitive ability and investment in defense against herbivores (Agrawal et al. 2012; Züst et al. 2012). Similar dynamics have been observed in natural systems: the trade-off between chemical defense and growth rate has been shown to be a greater contributor to habitat specialization than physiological constraints on utilizing nutrient poor growth environments (Fine et al. 2004). Insect herbivory has also contributed to plant trait changes over macroevolutionary time: an arms race with herbivores may have led to escalating chemical defenses throughout evolution in some plant groups (Becerra et al. 2009).

There is often specialization in both the herbivore and the plant. Perhaps due to plants' investment in a diversity of defense mechanisms, more than 90% of known insect herbivores feed on plants in three or fewer families (Bernays and Graham 1988), and specialization may become even more prevalent in the species-rich tropics ((Dyer et al. 2007) but see (Novotny and

Basset 2005)). In addition to comprising a majority of herbivore species, specialists are responsible for most of the observed damage from insect herbivory: in a study on Barro Colorado Island, 60% of herbivore-induced damage to study trees was caused by specialist insects, compared to just 8% by generalist insects and 32% by fungal pathogens (Barone 1998). In experimental studies, generalist females were worse than specialist females at discriminating high and low quality host plants, so specialist larvae may be better equipped to do damage to a chosen host plant (Janz and Nylin 1997). Furthermore, studies on specialized herbivores may be able to give us insight into insect herbivory and plant defense in general, since no consistent differences in plant responses to specialist versus generalist insects have been found when other differences between the insect herbivores were accounted for (Bidart-Bouzat and Kliebenstein 2011; Ali and Agrawal 2012).

The Passifloraceae-Heliconiine system is a promising model to understand the ecological and evolutionary dynamics of specialized herbivory. There is ample data on interactions between these groups extending back over 40 years due to interest in the system as well as ongoing scientific interest in the distinctive biology of *Heliconius* (compiled association/interaction data: (Robinson et al. 2010); ongoing interest in *Heliconius*: (Benson et al. 1975; Jiggins et al. 1996; Estrada and Jiggins 2002; Kronforst and Gilbert 2008; 2012; Bybee et al. 2012; Cardoso and Gilbert 2013)). Furthermore, the groups are quite specialized to one another -- non-Heliconiine herbivory of the Passifloraceae is limited to certain species of flea beetles and flag-legged coreid bugs (Gilbert and Smiley 1978; Pemberton 1983) --, but there is still substantial variation in the degree of species-level host specialization among Heliconiine herbivores. Herbivores in this system apply strong selective pressure to their hosts: in some cases a plant can be completely defoliated by a single pestiferous larva, leading to death, slow growth, or extended or permanent

juvenile state as a paucity of resources makes it impossible to invest in reproduction (Williams and Gilbert 1981; Gilbert 1982).

There is ample evidence of adaptation by *Passiflora* to avoid predation by *Heliconius*. *Passiflora* have evolved chemical defenses in the form of cyanogenic compounds (Smiley 1978a, 1985; Engler-Chaouat and Gilbert 2007), which may contribute to the avoidance of *Passiflora* by generalized herbivores (Denno and Donnelly 1981). Similarly, *Heliconius* species have evolved to cope with the myriad defenses of their hosts, demonstrating physiological and behavioral specialization to contend with host plant toxins and mechanical defenses (Cardoso 2008): Females prefer to lay their eggs on young parts of the plant, which tend to be poorly defended and nutrient-rich, and larvae that were fed young leaves in experimental studies showed increased survivorship and growth rate compared with larvae that were raised on older leaves (Rodrigues and Moreira 1999). Furthermore, *Heliconius* larvae have evolved mechanisms to reduce the release of cyanide gas from host plants while they eat compared to unspecialized insects (Helene et al. 2000; Alonso Amelot et al. 2006), and to sequester cyanogens from their host plants for their own defense or to synthesize them de novo (Engler-Chaouat and Gilbert 2007).

However, host choice may be an even more important factor than defense in maintaining particular plant-herbivore associations. Despite the sophistication of the physiological mechanisms that Heliconiine larvae have evolved to cope with the chemical defenses of Passifloraceae, evidence suggests that host choice by adult females is a greater contributor to the maintenance of specificity of host-pest associations in the Passifloraceae-Heliconiine system. Smiley's early studies (Smiley 1978a; Smiley 1978b) showed no difference in larval survival on host versus non-host *Passiflora* from La Selva (but see (Merrill et al. 2013)). Subsequent studies

showed no effect of chemical defenses (cyanogens, alkaloids, tannins, non-tanning phenols, saponins, or cardenolides) found in different *Passiflora* on larval growth; the only chemical character that affected larval growth rate was leaf nutrition content in the form of nitrogen concentration (Smiley and Wisdom 1985). There is also evidence that females exercise choice and modulation during host selection process: females laid fewer eggs per plant, but more eggs overall, when more host plants were available, and chose to lay fewer eggs on plants with conspecific eggs or larvae already present (Williams and Gilbert 1981). Host choice in Heliconiines has been found to mediate infestation at the community level as well (Copp and Davenport 1978a).

Olfactory and contact chemical cues are key modalities thought to facilitate the relationship between *Heliconius* and *Passiflora*. More chemosensory genes have been found in the *Heliconius* genome than in any other insect genome, suggesting the importance of chemosensation in this group (2012). In parallel, detailed studies have confirmed the role of contact chemical cues in *Heliconius* mate and host plant choice: after alighting on the leaf of a potential host, *Heliconius* females drum the surface of the leaf to taste for the appropriate chemical signals (Briscoe et al. 2013). Criucial roles for volatile cues in critical arenas like mating have also been found (Estrada et al. 2010). Nonetheless, there is little information on which airborne chemical compounds (volatiles) emitted from *Passiflora* vegetation are detected by the antenna. In addition, a comparison between *Passiflora* species' volatile profiles, in combination with the *Heliconius* species that are specialized for those *Passiflora* species, would provide a framework for understanding the evolution of hostplant defenses and pest sensory preferences.

Using techniques from phylogenetics, analytical chemistry, comparative biology, and insect neurophysiology, this study explores the role of volatile chemical cues in this specialized plant-herbivore system from the perspective of both plant and butterfly. Specifically, we ask what factors may contribute the evolution and maintenance of plant-herbivore associations, and how chemical cues have evolved across the *Passiflora*. We expected to find divergence in volatile chemistry between closely-related plant species, as species evolve to be inconspicuous to current herbivores, and presence of similar cues in the plant species used by a given herbivore. However, our results show that there is significant overlap in VOC profiles between *Passiflora* species, and *Heliconius* butterflies show antennal responses to similar suites of volatiles, suggesting this system is, at an evolutionary scale, more generalized than first appreciated.

MATERIALS AND METHODS

Materials and methods for plant rearing, voucher preparation, chemical analysis with gas chromatography/mass spectrometry, gathering and analysis of genetic data, and programming packages used for data processing and statistical analysis as in Clifford et. al. 2017 (Dissertation chapter 2; in submission, *Evolution*). The headspace sampling protocol was also the same, except that at 60 and 110 cm of leafy vine (typically 15+ leaves, except in species where leaves were very large) were cut from the plant for each scent sample.

Analysis of volatile headspace (scent) data used the same methods as Clifford et. al. 2017. However, we considered that the filtering method used for floral scents, where we required two or more samples to contain a given volatile, might be too punitive given the much lower emission rates of leaves relative to flowers; in the filtered data set many *Passiflora* species had

no detectable chemical profile over the controls and over half had 5 or fewer chemicals. To partially account for low chemical emissions approaching our limit of detection, we repeated all of the analyses on a less filtered version of the leaf headspace chemical data set, where compounds found in any of the samples of a given species were kept and averaged.

Insect Procurement and Rearing

Heliconius erato, H. melpomene, H. cydno, and H. hecale pupae were imported from Costa Rica through U.S.-based distributor LPS Imports under USDA APHIS interstate transport permit P526-140108-011 to MRC and JAR. These species were chosen because all co-occurred at J. Smiley's field site, where he well-characterized their relationships with *Passiflora* at that site and they represented a range of specialization in herbivory (Smiley 1978a; Smiley 1978b). After arrival, the pupae were sexed (Beebe et al. 1960; Gilbert 2015) and only females kept for experiments. Female pupae were taped by the ends of their abdomens to snap-close cup lids with no straw hole (SOLO, Dart Container Corporation, Mason, MI USA), and enclosed in a cup with a moist paper towel until eclosion; if pupae did not emerge after two weeks or showed fungal growth or presence of parasites, the cups were autoclaved without opening. After emergence, butterflies were kept sex-segregated in parasitoid-resistant mesh flight cages and fed with Gatorade sports beverage soaked into a paper towel (PepsiCo, Purchase, NY, USA) sprinkled with crushed corn pollen (The Green Spot Ltd.), replaced daily (Cowan 2015).

Heliconiine phylogeny

We extracted the large Heliconiine phylogeny from the latest available study(Kozak et al. 2015) using TreeSnatcher software (Laubach et al. 2012). We used the resulting machine-readable Newick-format phylogeny in all analyses accounting for Heliconiine relatedness.

Electrophysiology and Olfactory Stimulus Presentation

Antennal preparation: Electroantennograms (EAGs) and gas-chromatograph-coupled electroantennogram detection (GC-EADs) were performed using apparati and procedures as in (Byers et al. 2014). Insect antennae were prepared by dissecting them from the insect and removing the distal tip with tenotomy scissors. One antenna was used for each preparation. The proximal end of the antenna was inserted into a Spectra 360 electrode gel-filled (Parker Labs, Fairfield, NJ, USA) glass pipette tip, and the distal end was punctured by a pulled-tip glass pipette to preserve the OSN-rich tip of the antenna (Briscoe et al. 2013). These were placed between two silver chloride electrodes constructed using silver wire stripped of its teflon coating (bare: 0.0050", A-M Systems, Sequim, WA, USA) so that the electrodes could measure electrical activity moving across the antenna(e) (as in (Andersson and Dobson 2003a; Fraser et al. 2003)). The electrodes were connected to a headstage (A-M systems), chained into a 1800 AC amplifier (A-M Systems), then to a noise reducing device (Humbug Noise Eliminator; A-M Systems), and then into an RZ2 amplifier (Tucker-Davis Technologies, Alachua, FL, USA). The electrodes and antenna(e) were placed in front of a continuous stream of air (100 mL/min flow; Gilmont flowmeter, Gilmont Industries/Barnant Company, Barrington, IL, USA) at room temperature.

GC-EAD chemical stimuli and analysis: For GC-EADs, a GC-FID (Agilent 7820A GC with Flame Ionization Detector, Agilent Technologies; DB5 column, J&W Scientific, Folsom, CA, USA) was used with a glass y-splitter was used to present half of the GC effluent to the

antenna(e) as the chemical stimulus, to allow half to proceed to the FID detector; chemicals were presented in the order and concentration that the emerged from the GC. The results were recorded with WinEDR software (Windows Electrophysiology Disk Recorder V3.7.3, Strathclyde Electrophysiology Software, copyright John Dempster 1996-2017), which recorded GC-FID and corresponding EAD traces in the a single file with the same timestamps. GC-FID traces were aligned between samples using cross-correlation in R and then hand-checked. Because of contaminant peaks in some cases, hand-alignment was required and was performed using the locator function on focal peaks in R. The same alignment time lags applied to a GC-FID sample were applied to its corresponding GC-EAG trace. After alignment, normalized EAD responses (absolute value of zscore,/ maximum per preparation) corresponding to the leaf scent of given Passiflora species were divided into 5 second time bins (max value within time bin taken). These values were used as inputs into PCA and PERMANOVA. Sample sizes for GC-EADs in Table 4.

EAG chemical stimuli and analysis: For EAGs, chemical stimuli were presented using an odor cartridge, constructed from a 2 mL glass syringe (Air-Tite Products Co., Virginia Beach, VA, USA) and standard 20G 1 inch needle (PrecisionGlide; Becton, Dickinson, and Company; Franklin Lakes, NJ, USA) connected to a piece of plastic tubing with teflon tape to affix it to the stimulus line. All stimuli were pipetted onto a small piece of filter paper (Whatman Inc., Clifton, NJ, USA) in the odor cartridge. Vegetative sample consisted of all of the leaves on a length of vine between 60 and 110 cm in length sampled for 24 hours before eluting into 800 µL of hexane; 100 µL was used for each experiment . The odor cartridge was connected to its own line with air flowing at 10 mL/min; stimulus presentation was controlled with a solenoid activated by OpenEx software.

Responses were recorded to each stimulus and analyzed in R. Sample sizes for EAGs were: 7 *H. cydno*, 7 *H. erato*, 8 *H. hecale*, 9 *H. melpomene* individuals.

Controls: Negative controls consisted of 100 µL hexane, to control for the amount of hexane in the vegetative samples, and 5 µL mineral oil, which does not volatilize and should control for any antennal response solely to mechanical stimulation from the puff of air accompanying stimulus delivery. Positive controls consisted of 5 µL (±)linalool, a common floral volatile known to elicit large antennal responses in *Heliconius* butterflies (*H. melpomene*: (Andersson and Dobson 2003a)). Antennae were used only if the preparation was responsive to linalool. Controls were presented at the beginning and end of the experiments.

Plant Scent Stimuli: Six *Passiflora* species were used for leaf scent in the odor panel; these species were chosen because their associations with chosen *Heliconius* species were well-characterized at the La Selva field site (Smiley 1978a; Smiley 1978b).

Because most butterfly species showed strong differences in the magnitude of responses between individual preps, we tested for differences in responses to host- and non-host-plant odors using linear mixed effects models, which fit the data better or as well as than models accounting for the fixed effect of odor class alone (linear model, SD EAG response ~ odor class) or for the random effect of individual prep alone (linear mixed effect model, SD EAG response ~ (1|individual prep)) according to AIC values for both (Table 1) and likelihood ratio tests for the mixed effects models, Pr(Chi-square) <<< 0.001 for all butterfly species) (likelihood ratio test: (Pinheiro 2000; Bates et al. 2015; Pinheiro et al. 2016)).

RESULTS

Passiflora leaf scent chemistry

In the more filtered chemical data (n = 70 *Passiflora* spp), 9 species had no detectable chemicals in their leaf headspace (over controls), while 61 had a detectable chemical profile; in total, 230 distinct volatiles were found in these leaf scents. In the less filtered chemical data set (n = 70 *Passiflora* spp), only one species, *P. bryonioides*, had no detectable chemical profile relative to controls; using this standard, 614 distinct volatiles were found in these *Passiflora* leaf scents. The most common chemicals across sampled species were largely consistent between the more and less filtered data sets (more filtered; less filtered): beta-ocimene (38 spp.; 57 spp.), caryophyllene (30 spp; 47 spp.), ethyl benzoate (28 spp.; 46 spp.), (E)-beta-farnesene (20 spp.; 36 spp.), alpha-farnesene (20 spp.; 34 spp.), ethyl salicylate (19 spp.; 37 spp.), cubenene (19 spp.; 39 spp.), ethyl hexanoate (15 spp.; 33 spp.), ylangene (15 spp.; 32 spp.), linalool (14 spp.; 26 spp.), nerolidol (14 spp.; 32 spp.), bisabolene (13 spp.; 24 spp.), supraene (13 spp.; 26 spp.), cis-alpha-bergamotene (12 spp.), 3-hexen-1-ol butanoate (12 spp.; 26 spp.), piperidine (11 spp.; 23 spp.), and germacrene D (10 spp.; 24 spp.). In the more filtered data set, the remaining 213 volatiles were found in the headspace of nine or fewer of the included *Passiflora* species; in the less filtered data set, the remaining 597 volatiles were found in 21 or fewer of the included *Passiflora* species, and 540 volatiles found in 9 or fewer species. Both the more and less filtered data sets contained primarily terpenes and their derivatives, as well as carboxylic acids and oxygenated alkanes. Both also contained the alkaloid piperidine and its derivatives.

Leaf scent chemistry, species associations, and locality

To investigate the role of leaf scent chemistry in *Heliconius-Passiflora* associations, we examined the relationship between pairs of species with or without existing associations. The bulk of the following statistical tests were done with both filtered and less filtered versions of the leaf chemical headspace data set with very similar results (we did not repeat the labor-intensive analyses regarding chemical class, eg. number of terpenes or Mantel tests with terpenes only, for the less filtered data after seeing the similarity of all other tests with the more filtered data); results are shown for the more filtered data in this section given these similarities. Tests for correlation between leaf scent chemistry and herbivore association, both with and without accounting for plant phylogeny, showed no evidence of a relationship between the two (Figure 1B-1D; n = 37; Mantel test, raw leaf chemistry ~ herbivore association: Mantel r = 0.00042, significance = 0.458; Mantel test, raw leaf chemistry ~ plant phylogeny + herbivore association: Mantel r = -0.000102, significance = 0.48). Similarly, using data on the presence/absence of chemicals in each leaf scent rather than the chemical concentrations showed no significant relationship between leaf VOCs and herbivore associations (n = 37; Mantel test, presence/absence leaf chemistry ~ herbivore association: Mantel r = 0.009, significance = 0.432 ; Mantel test, presence/absence leaf chemistry ~ plant phylogeny + herbivore association: Mantel r = 0.003, significance = 0.446). Interestingly, plant-relatedness also did not appear to explain leaf scent chemistry (Figure 1A; n = 37; Mantel test, raw leaf chemistry ~ plant phylogeny: Mantel r = 0.0412, significance = 0.971; Mantel test, presence/absence leaf chemistry ~ plant phylogeny: Mantel r = -0.067, significance = 0.878). Other computational approaches also did not demonstrate evidence of any relationship between leaf scent chemistry and herbivore association: for instance, a bipartite network similarity clustering approach (as in Vilhena et al

2014) on leaf scent was not able to distinguish between *Passiflora* species herbivorized by different butterfly species.

Similar tests on subsets of chemical data (chemical abundance, proportions, and presence/absence) containing terpenes, a class of chemical known as to be used as plant defense compounds (eg, caryophyllene, ocimene, farnesene (Unsicker et al. 2009), also showed no relationship between herbivory and leaf scent, with or without accounting for plant relatedness (n = 37; Mantel test, raw terpene chemistry ~ herbivore association: Mantel r = 0.02, significance = 0.302; Mantel test, raw terpene chemistry ~ herbivore association + plant phylogeny: Mantel r = 0.021, significance = 0.296; Mantel test, raw monoterpene chemistry ~ herbivore association, Mantel r = -0.024, significance = 0.658; Mantel test, raw sesquiterpene chemistry ~ herbivore association, Mantel r = -0.009, significance = 0.542). Similarly, there was no relationship between which defense compounds were present and which herbivore associations a plant species had, with or without taking plant relatedness into account (n = 37; Mantel test, presence/absence terpene chemistry ~ herbivore association + plant phylogeny: Mantel r = -0.0069, significance = 0.494; Mantel test, presence/absence terpene chemistry ~ herbivore association: Mantel r = -0.0032, significance = 0.482). The number of non-terpene compounds known to be used in plant defense (eg. phenolics: 2; nitrogenous compounds: 4) was so small and sparsely emitted by *Passiflora* species in our data set that these compounds were unlikely to explain associations with phytophagous butterflies across the group.

The diversity and number of individual chemicals present in leaf scent may also be a factor involved in the evolution of the chemical profile of *Passiflora* vegetation; we therefore examined this in relation to the *Heliconius* relationship. Our findings show no significant relationship between number of chemicals in leaf scent for a given plant species and its number

of associations with phytophagous butterflies, when not taking phylogeny into account (n = 37; Poisson generalized linear model for count data with log-link, number of herbivore associations ~ number of chemicals in leaf headspace, McFadden's pseudo R^2 = 0.0034, glm effect estimate = -0.007, p = 0.274) and a small, negative relationship when taking phylogeny into account (Poisson phylogenetic generalized linear model, number of herbivore associations ~ number of chemicals in leaf headspace, glm effect estimate = -0.064, p(Wald's) = 0.0377) that disappeared when *Passiflora* species with no detectable chemicals in the leaf headspace (relative to controls) were removed from the analysis (Poisson phylogenetic generalized linear model, number of herbivore associations ~ number of chemicals in leaf headspace with zeroes removed, glm effect estimate = -0.040, p(Wald's) = 0.1389). Overall intensity of the scent was not explained by the number of herbivore associations when not taking phylogeny into account (n = 37; linear model, number of herbivore associations ~ $\log_{10}$(chemical intensity), effect estimate = -0.027, p = 0.529); although there was a negative trend between chemical intensity and number of herbivore associations when taking phylogeny into account (phylogenetic linear model, number of herbivore associations ~ $\log_{10}$(chemical intensity), effect estimate = -0.173, p = 0.116), but this relationship vanished when *Passiflora* species with no detectable leaf scent (relative to controls) were removed (phylogenetic linear model, number of herbivore associations ~ $\log_{10}$(chemical intensity) with species with zero chemical emission removed, effect estimate = -0.0014, p = 0.981). Similarly, there was no relationship between the number of individual terpenes that were emitted by a particular plant species and its quantity of herbivore associations (n = 37; Poisson generalized linear model for count data with log-link, number of herbivore associations ~ number of terpenes in leaf headspace, McFadden's pseudo R^2 = 0.0044, glm effect estimate = -0.007, p = 0.518) when plant phylogeny was not accounted for. When plant phylogeny was

explicitly taken into account, (n = 37, Poisson phylogenetic generalized linear model, number of herbivore associations ~ number of terpenes in leaf headspace, glm effect estimate = -0.055, p (Wald's) = 0.0615), the test yielded an almost significant p-value, but a tiny and negative effect size for the number of terpenes in a plant species leaf headspace relative to the number of association it had with herbivores.

Leaf volatile chemistry (chemical abundance, proportions, and presence/absence) was also not explained by which locations *Passiflora* species were found based on aggregated field observations in the HOST database (Robinson et al. 2010), with the caveats that these records are not exhaustive; that we were not able to actually sample individuals from these different localities, and leaf scent for a given species may vary based on locality; and that our tests treat locations as independent rather than considering their relationship to one another in space or similarity in habitat type, climate, or any biotic variables such as Heliconiine herbivore diversity (Rosser et al. 2012) (n = 37; Mantel test, raw leaf chemistry ~ locations found (factor): Mantel r = 0.034, significance = 0.207; Mantel test, raw leaf chemistry ~ plant phylogeny + locations found (factor): Mantel r = 0.034, significance = 0.177; Mantel test, presence/absence leaf chemistry ~ locations found (factor): Mantel r = 0.040 , significance = 0.201; Mantel test, presence/absence leaf chemistry ~ plant phylogeny + locations found: Mantel r = 0.039, significance = 0.228).

Phylogeny and species associations

About 30% of the variance in plant-butterfly associations was explained by phylogenetic relatedness between *Passiflora* species (Figure 2B; n = 76; Mantel test, plant-insect association ~ plant phylogeny: Mantel r = 0.2942, significance = 0.001). However, we did not find evidence of

a concomitant relationship between plant-butterfly associations and relatedness between butterfly species (Figure 2A; n = 45; Mantel test, plant-insect association ~ insect phylogeny: Mantel r = -0.06126, significance = 0.814). These results suggest that herbivores feed on related plants, but that related herbivores do not have the similar host plant preferences. This may suggest diversification in host plant preference on the part of predaceous Heliconiians, rather than co-evolution of the plants and butterflies in tandem, which has advanced in the past to explain this system ((Benson et al. 1975; Marquis et al. 2016); but see (Bernays and Graham 1988; Jaenike 1990; Suchan and Alvarez 2015)).

Heliconius EAG response to the leaf scent of host and non-host plants

We tested the antennal response of *Heliconius* species (*H. erato*: n = 7, *H. hecale*: n = 8, *H. melpomene*: n = 9, *H. cydno* = 7) to the leaf scent extracts of host and non-host *Passiflora* species, known from field studies at La Selva Biological Station in Costa Rica (Smiley). All butterfly species showed significantly higher responses to the positive control than to the negative controls (Figure 3B). However, though the two more specialized species, *H. erato* and *H. melpomene*, showed significantly higher responses to both host and non-host leaf odors than to the negative control, *H. hecale* and *H. cydno* did not. None of the butterfly species showed a significantly higher response to leaf odors from host plants than to leaf odors from non-host plants (Figure 3; linear mixed effect model, SD EAG response ~ odor class + (1|individual prep); coefficient estimates and significance, Table group 2, and 95% confidence intervals for effect sizes, Table group 3).

Heliconius GC-EAG responses to individual volatiles from host and non-host plant scents

Using the same sets of *Heliconius* and *Passiflora* species as those included in the EAG experiments, we used GC-EAGs to measure the antennal response of butterfly species to the individual chemical constituents of leaf scent (GC-EAG sample sizes: Table 4). These experiments tested for sensory filtering of the scents by associated species, and for butterfly species- or herbivory-association-specific patterns of response to scent components that may not have been possible to discriminate using the bulk antennal responses. We did not find evidence that herbivory association or butterfly species explained patterns of antennal response to chemical constituents for any tested plant species (PERMANOVA; Table 5), though there may be consistency in responses within butterfly species with larger sample sizes. Though relationships were all insignificant, larger effect sizes suggested that butterfly species may explain a greater proportion of response variation than whether or not the butterfly species used the plant as a host, which explained almost none of the variation in response.

DISCUSSION

Passiflora leaf volatile profiles and Heliconiine chemosensation

Leaf scent chemistry in the *Passiflora* was dominated by terpenes and their derivatives, carboxylic acids, and the alkaloid piperidine. Many of these volatiles are known from other systems to play a role in plant defense, whether by directly repelling herbivores or indirectly, by signaling the presence of prey to their herbivores' predators or parasitoids (Kessler and Baldwin 2001; Unsicker et al. 2009). Although the *Passiflora* are known to use cyanogenic glycosides (Smiley 1985; Alonso Amelot et al. 2006) as an inducible defense, we did not find evidence that cyanogenic glycosides themselves or hydrogen cyanide they produce during herbivory were

constitutively emitted by the leaf tissue, though it is possible that any hydrogen cyanide emission was obscured by the solvent peak. Unexpectedly, we also found that plant relatedness did not explain variation in leaf scent chemistry, suggesting that genetic drift was not the lone factor in shaping the volatile profile of a given *Passiflora* species.

Moving beyond the characterization of the leaf scent chemistry itself, we tested whether leaf scent chemistry explained the herbivory associations found in field data, as we would expect if leaf scent were being used by herbivores used to find their host plants. However, using a variety of approaches, we did not find evidence of any relationship between leaf scent chemistry and herbivory associations. Furthermore, using both EAG and GC-EAG methods, we found no evidence of differential sensory responses by *Heliconius* species to host versus non-host leaf scent.

Sensory integration in Heliconius for hostplant discrimination

Beyond olfactory cues, *Heliconius* species are known to utilize visual cues and contact chemical cues to motivate critical functions. *Heliconius* has been shown to use color to choose which flowers to feed on, and are able to more readily associate color than scent with feeding (Andersson and Dobson 2003b), as with many other butterfly species. In species that exhibit pupal mating, in addition to using volatile cues (Estrada et al. 2010), males have been observed to visually inspect hanging objects on host plants (Gilbert 1982) as part of their search for pupal females. And, with implications for their broad biological importance in this group, visual cues have played a critical role in mate choice evolution in the genus. UV wing coloration evolved concurrently with UV sensitive opsins in the *Heliconius*; their predators, however, did not evolve the ability to see these wavelengths (Briscoe et al. 2010; Bybee et al. 2012). The evolution of this

private channel may reduce the cost of mimicry, allowing individuals to identify conspecifics, while still mimicking co-locating species to their predators (Bybee et al. 2012).

Because of their accessibility or because of their noted biological importance in this group, visual cues have historically received greater attention as mediators of host choice and evolution in this system. In early literature, Larry Gilbert and colleagues speculated about the coevolution of *Heliconius* and *Passiflora,* and the potential role of the use of search images by the visually acute *Heliconius* in selecting for diverging leaf shapes in the *Passiflora* (Benson et al. 1975; Gilbert 1982), as found in other plant-butterfly systems (Rausher 1978). Though direct evidence for *Heliconius*-mediated selection driving the diversification of leaf shape in *Passiflora* has not been found, it is not implausible given the importance of visual cues to *Heliconius* species in other arenas. Indeed, certain visual cues have been found to mediate both innate and learned responses that feed into host plant search and choice. Females chose to layer fewer eggs on plants with egg spots, likely an exclusively visual cue (Williams and Gilbert 1981). Abrams and Gilbert found that young *Heliconius* females were attracted to wire models of tendrils, suggesting an innate ability to visually distinguish vine from non-vine plants; the same group found that older *Heliconius* females approached and investigated plants that resembled their host plant's overall appearance and leaf shape, implying a role of visual learning in host plant choice (Gilbert 1982). Even more directly, a recent study showed that *Heliconius* can learn to associate leaf or flower shape with reward, and that this can affect their oviposition preference (Dell et al. 2016).

Detailed studies have also confirmed the role of contact chemical cues in *Heliconius* mate and host plant choice. In species with pupal mating, males use contact chemical cues to discriminate female from male pupae, as well as to gauge pupal maturity (Estrada et al. 2010). In

species where females typically mate only once while males mate multiple times, males use a contact chemical 'antiaphrodesiac' to avoid wasting resources on pursuing a mated female (Estrada 2011). There is also strong evidence for the use of leaf surface chemicals in the final steps of host plant choice. After alighting on the leaf of a potential host, *Heliconius* females drum the surface of the leaf to taste for the appropriate chemical signals, a common process in many butterfly species (Gilbert 1982; Briscoe et al. 2013). In *Heliconius,* expression of gustatory receptors involved in this process are enriched in the female chemosensory tissues of antennae, legs, palps, and proboscis, but not in those of males, sensory sexual dimorphism that suggests the importance of these receptors in the female-specific task of host plant finding (Briscoe et al. 2013).

Taken together, our results suggest that volatiles may not be important cues for host plant finding in the *Heliconius-Passiflora* system, and that other cues may be more salient in this system despite many examples in the Lepidoptera where volatile chemicals are used for this purpose (eg. *Manduca, Cydia*; (Najar-Rodriguez et al. 2010)). Alternatively, it could be sensory integration plays an important role in host-finding in this system, i.e., that leaf volatiles alone are not sufficient to discriminate host- from non-host-plants, and instead serve as a more generic indication of a potential host plant that must be used in tandem with subsequent visual and/or gustatory cues to make the final choice. There is behavioral evidence for host plant navigation using chemical cues that support this idea: Heliconiine *Agraulis vanillae* was observed to fly upwind to visually obscured host plants in experimental trials (Copp and Davenport 1978b), in a manner similar to *Manduca sexta* and other pestiferous moths (REFS). And, in experiments, others have observed female Heliconiines alighting on *Passiflora* leaves, drumming them to test the gustatory cues, and then choosing not to lay eggs on undesirable plants (Thiele et al. 2016).

There is also evidence that *Heliconius* use olfactory cues in tandem with visual cues in other critical arenas like foraging and mating (eg. floral scent is used along with color to determine which flowers to pollinate (Andersson and Dobson 2003)). The hypothesis of a generic role of leaf scent in host plant finding could be tested by seeing if Heliconiines fly upwind to *Passiflora* versus non-*Passiflora* plants when visual cues are obscured.

Future work: possible roles of phylogeny and geography in Passiflora-Heliconiine associations and addressing gaps in this study

The influence of geography and phylogeny on *Passiflora*-Heliconiine associations are an area of potentially fruitful future work. Though we did not have data on possible confounds (eg. spatial and temporal sampling effort, site-specific characteristics, etc.) in geographic and plant-herbivore association data, simple tests showed a strong relationship between the number of locations that a species was found and the number of associations it had. For *Passiflora*, the number of sites a given species was found explained about 30% of the variance in the number of associations it had with herbivores (n = 114, Poisson generalized linear model for count data with log-link, number of herbivore associations ~ number of locations found, McFadden's pseudo $R^2 = 0.27$, glm effect estimate = 0.161, intercept = 0.866, $p = 2 \times 10^{-16}$), both with and without taking plant relatedness into account (n = 76, Poisson phylogenetic generalized linear model, number of plant associations ~ number of locations found, glm effect estimate = 0.156, intercept = 1.237, p (Wald's) = 0). For *Heliconius*, the effect was even stronger, explaining about 80% of the variance (n = 51, Poisson generalized linear model for count data with log-link, number of plant associations ~ number of locations found, McFadden's pseudo $R^2 = 0.775$, glm effect estimate = 0.152, intercept = 0.964, $p = 2 \times 10^{-16}$), with a similar effect size both with and without taking insect relatedness into account (n = 45, Poisson phylogenetic generalized linear

model, number of plant associations ~ number of locations found, glm effect estimate = 0.101, intercept = 2.09, p (Wald's) = 0). This may suggest that *Heliconius* butterflies may be limited in the hosts that they can use by their geographic range; when they were found at more sites, they fed on more plant species. *Passiflora* species appeared to be more limited in the number of sites they colonized and in the number of associations with herbivores. However, future work should quantify range more explicitly and account for possible confounds to better test this possibility in this system. Furthermore, we found that 30% of variation in plant-herbivore associations was explained by Passiflora relatedness, but that no such relationship existed between plant-herbivore associations and Heliconiine relatedness. This suggests that Heliconiine herbivores tend to feed on plants that are more closely related to one another, but that related herbivores do not have more similar host plant preferences. More work should be done to elucidate the mechanism for this relationship as well.

Future work could also address gaps in the chemical aspect of this study. It is possible that leaf scent is plastic, or alternatively, that geographic or individual variation outstrips variation in leaf scent between *Passiflora* species. Such plasticity and geographic variation has been found in floral scent in some systems and would make sense for leaf headspace, as different locales may have different biotic defense needs. Each of these possibilities could contribute to not finding an association between leaf scent chemistry and plant-herbivore association, and, if there was a mismatch in the provenance locale of the plant species and the butterfly species, to not finding an a difference in sensory response to host versus non-host plants. Whether leaf scent is plastic in this system could be disambiguated by growing the plants under experimentally different conditions and assessing differences in leaf scent emission. Individual variation could be better quantifying by including larger numbers of genetically distinct individuals for a given

Passiflora species, preferably wild-collected given the unknown provenance of many cuttings and seed available through the greenhouse/collector trade. Geographic variation could be quantified by collecting leaf headspace from multiple individuals of the same species across more than one locale; corresponding electrophysiology or behavioral experiments with insects from the same locale could determine if host-discrimination using chemical cues occurs or if cues from other modalities are hard required. Understanding the plasticity and individual and geographic variation could help elucidate the role of chemical cues in this system.

ACKNOWLEDGMENTS

We thank J. MacDougal and L. Gilbert for providing botanical samples and herbivory observations, assistance with species identification, and for fruitful discussion on the literature and phylogenetic context; R. Cowen and L. Gilbert for advice on *Heliconius* care and sexing, R. Olmstead for providing supplies for molecular analysis; D. Giblin and M. Yousoufian for help with mounting and cataloguing voucher specimens; G. Wisswaesser for his aid with plant maintenance and with collecting samples and vouchers; and D. Ewing, N. Kurashige, J. Milne, P. Beeman and the UW Greenhouse staff for fruitful discussions, plant care, and for being instrumental in finding methods to get many species to germinate or take root. This work was supported by NSF DGE-0718124, Riddiford-Truman Fellowship, and UW BEACON grant to MRC.

TABLES

Table 1: AICs for comparisons between candidate models for EAG responses to leaf odors. Comparisons between the chosen model (linear mixed effects model, fixed effect = odor type, random effect = individual preparation) and LMEM with random effect of individual prep. alone and LM with odor type alone. In all cases AICs for the chosen model were more favorable (lower) or approximately equal to the other models.

	LMEM(chosen, both)	**LMEM(indiv. only)**	**LM(odor type only)**
H. erato	293.53	325.99	313.17
H. hecale	320.89	358.73	323.63
H. melpomene	360.36	422.24	401.83
H. cydno	282.52	343.32	280.52

Table 2: Table group of effect estimates and significance values for linear mixed effects models (LMEM) for EAG response against leaf odor type + individual prep identity. Intercept is negative control estimate tested against zero value. All others are estimate of effect size relative to negative control. Values are based on the normalized response per individual using a z-score for each stimulation against the mean of the whole trace. Half of butterfly species had significantly larger antennal responses to leaf odors than negative controls, but half did not.

(a) H. erato

	Estimate	**Std. Error**	**t-value**	**p-value**
(Intercept)	1.86	0.54	3.43	<<< 0.0001***
Non-Host Plants	1.93	0.42	4.55	<<< 0.0001***
Host Plants	2.61	0.52	5.03	<<< 0.0001***
Pos. Control	4.26	0.	6.71	<<< 0.0001***

(b) H. hecale

	Estimate	**Std. Error**	**t-value**	**p-value**

(Intercept)	2.71	0.38	7.09	<<< 0.001***
Non-Host Plants	0.81	0.47	1.71	0.087
Host Plants	0.81	0.39	2.09	0.037*
Pos. Control	4.28	0.58	7.39	<<< 0.001***

(c) H. melpomene

	Estimate	**Std. Error**	**t-value**	**p-value**
(Intercept)	1.93	0.50	3.84	0.0001***
Non-Host Plants	1.19	0.34	3.48	0.0004***
Host Plants	1.29	0.42	3.08	0.002**
Pos. Control	5.09	0.51	9.92	<<< 0.001***

(d) H. cydno

	Estimate	**Std. Error**	**t-value**	**p-value**
(Intercept)	2.67	0.38	7.04	<<< 0.001***
Non-Host Plants	NA	NA	NA	NA
Host Plants	0.40	0.42	0.93	0.35
Pos. Control	5.97	0.66	9.07	<<< 0.001***

Table 3: Table group of 95% confidence intervals for linear mixed effects models (LMEM) for EAG response against leaf odor type + individual prep identity. Intercept is negative control estimate tested against zero value. All others are estimate of effect size relative to negative control. Values are based on the normalized response per individual using a z-score for each stimulation against the mean of the whole trace. No butterfly species showed a significant difference in response to leaf odors from host- versus non-host plant species.

(a) H. erato

	2.5%	**97.5%**

(Intercept)	0.73	2.99
Non-Host Plants	1.09	2.77
Host Plants	1.58	3.64
Pos. Control	3.00	5.53

(b) H. hecale

	2.5%	**97.5%**
(Intercept)	1.95	3.48
Non-Host Plants	-0.13	1.75
Host Plants	0.04	1.57
Pos. Control	3.13	5.43

(c) H. melpomene

	2.5%	**97.5%**
(Intercept)	0.88	2.98
Non-Host Plants	0.51	1.87
Host Plants	0.46	2.12
Pos. Control	4.08	6.12

(d) H. cydno

	2.5%	**97.5%**
(Intercept)	1.92	3.42
Non-Host Plants	NA	NA

Host Plants	-0.45	1.24
Pos. Control	4.66	7.27

Table 4: Sample sizes for GC-EAGs; Passiflora *species are column names and* Heliconius *species are row names.*

	amb.	**bifl.**	**cor.**	**cost.**	**meni.**	**oer.**	**quad.**	**vit.**	**TOTAL**
H. erato	4	4	4	3	5	3	4	4	31
H. cydno	2	2	2	2	2	2	2	1	15
H. melp.	4	4	4	4	2	3	1	3	25
H.hecale	3	4	3	4	4	4	3	3	28
TOTAL	13	14	13	13	13	12	10	11	**99**

Table 5:p-values (and effect size, R2) of PERMANOVAs on GC-EAG data show (1) no evidence for Heliconius-species-specific patterns of antennal response to individual compounds emitted by the plant leaves, and (2) no significant differences in antennal responses to host versus non-host plants. Though relationships were all insignificant, effect sizes (in parentheses) suggested that butterfly species identity may explain a greater proportion of response variation than whether or not the butterfly species used the plant as a host, which explained almost none of the variation in response. .

	amb.	**bifl.**	**cor.**	**cost.**	**meni.**	**oer.**	**quad.**	**vit.**
between Heliconius spp.	0.64 (0.21)	0.25 (0.29)	0.87 (0.16)	0.65 (0.20)	0.22 (0.31)	0.31 (0.30)	0.42 (0.35)	0.63 (0.26)
between Hel. spp. that use plant as host and those that do not	0.54 (0.06)	0.81 (0.04)	0.53 (0.06)	0.70 (0.05)	0.44 (0.08)	0.46 (0.08)	0.70 (0.06)	0.76 (0.06)

Table 6: Locations from Benson et. al. 1975 field studies documenting associations between Heliconiines and Passiflora species; letter keys used in figure 5.

Location (Benson et al. 1975)	**Letter Key**

Extreme southern Texas to Mexico to Nicaragua, northern Neotropics (Guatemala)	A
Jamaica and other West Indian islands	B
Panama and southeastern Costa Rica (Chiriqui, Darien)	C
Northern Venezuela (Rancho Grande)	D
Trinidad (eastern part of Sucre/Trinidad)	E
Northwestern Colombia, area of Quibdo (Choco)	F
Foothills and Ilanos in Orinoco drainage of eastern Colombia (Villavicencio)	G
Central Colombian valleys (Cauca, Magdalena)	H
Napo drainage in eastern Ecuadorian Andes (Abitagua, Napo)	I
Western Ecuador (Chimborazo)	J
Eastern Peru *Ucayali, Loreto, Chanchamayo)	K
Extreme northern Brazil (Roraima, Parima)	L
Guyana, Surinam, and (French) Guyane (Oyapock, northern part of Manaus/Guiana)	M
Lower and middle Amazon area in Para and eastern Amazonas, Brazil (Belem, Tapajos, southern part of Manaus/Guiana)	N
Lima area of western Peru south to northern Chile	O
Northeastern Bolivia (Yungas)	P
Southwestern Brazil, western Mato Grosso and Rondonia (Guapore, Rondonia)	Q
Central Brazil, eastern Mato Grosso and Goias (Araguaia)	R
Eastern Brazil, Pernambuco through Bahia to northern Espirito Santo (Bahia)	S
Southeastern Brazil, Minas Gerais, Rio de Janeiro and Sao Paulo (Rio de Janeiro)	T
Extreme southern Brazil, Santa Catarina and Rio Grande do Sul	U

FIGURE LEGENDS

Figure 1: Leaf volatile chemistry shows diversity that is not explained by plant associations with phytophagous butterflies

(A) NMDS of raw leaf volatile chemistry versus *Passiflora* subgenus

(B) NMDS of raw leaf volatile chemistry versus number of associations with herbivorous butterfly species

(C) NMDS of raw leaf volatile chemistry with examples of associations with selected butterfly species

(D) Leaf volatile chemistry was diverse among species of *Passiflora*; from top to bottom, chromatograms showing chemical peaks over time from (1) *P. ambigua*, (2) *P. biflora*, (3) *P. coriacea*, (4) *P. costarricensis*, (5) *P. menispermifolia*, (6) *P. oerstedii*, (7) *P. vitifolia*.

Figure 2: Global associations between Passifloraceae and Heliconiine butterflies

(A) *Heliconius* phylogeny against plant-insect associations; closely related butterfly species have dramatically different associations with host plant species.

(B) *Passiflora* phylogeny against plant-insect associations; about 30% of the variation in host plant – insect associations are explained by relatedness between plant species.

Figure 3: *Heliconius* EAGs to whole leaf odors from host and non-host *Passiflora* species show no significant differences in antennal response. Well-studied associations from intensive field studies at La Selva Biological Station in Costa Rica were used.

(A) *Heliconius* species used in electrophysiological assays. These species co-occur at La Selva, and *H. erato* and *H. melpomene* are part of a mimicry ring throughout their habitat ranges.

(B) Linear mixed effects models showed no cases where EAG response was higher to leaf odors from host plant species than non-host plant species.

(C) LMEM results for EAG responses to odors that made up negative control, herbivory association, no association, and positive control groups. Groupings varied among butterfly species because of associations with different host plant species at La Selva.

Figure 4: GC-EAGs show (1) no evidence for Heliconius-species-specific patterns of antennal response to individual compounds emitted by the plant leaves, and (2) no significant differences in antennal responses to host versus non-host plants. The same well-studied associations from intensive field studies at La Selva Biological Station as were used for EAGs were used for these GC-EAGs.

(A) PCAs of GC-EAG responses by *Passiflora* species, comparing *Heliconius* butterfly species that have an herbivory association with that plant species (green) against those that did herbivorize that plant species (purple), but were found in the same area. For none of the plant species did we find a significant difference in antennal response based on herbivory status (Table 5)

(B) A matrix of *Passiflora* host-plants and associated *Heliconius* butterfly species based on Smiley's study in La Selva. Green denotes a host plant association, purple denotes no known association between plant and butterfly species in La Selva.

(C) Example of a GC-FID trace of leaf scent headspace (*Passiflora ambigua*) and corresponding EAG responses (by *Heliconius hecale*). Chemical peaks with corresponding responses to (a) linalool, (b) ethyl benzoate, and (c) nerolidol.

Figure 5: Potential future work – Preliminary analysis based on data from Benson et al. 1975 shows that number of locations found explains the bulk of variation in how many host plants an

Heliconiine has, and a lesser, but still significant amount of the variation in how many herbivore a *Passiflora* species has.

(A) Map of locations from Benson study, and example of Heliconiine and Passiflora species found in location C (Panama and southeastern Costa Rica (Darien, Chiriqui)). See Table 6 for more information on locations.

(B) Species by location with phylogenies for Heliconiine butterflies (top) and *Passiflora* (bottom). Locations found was not explained by phylogeny for either butterflies (n = 43, Mantel test locations found for butterflies ~ butterfly phylogeny, r = 0.08, p = 0.16) or plants (n = 37, Mantel test locations found for plants ~ plant phylogeny, r = -0.01, p = 0.52).

(C) For *Passiflora* (bottom), the number of sites a given species was found explained about 30% of the variance in the number of associations it had with herbivores (n = 114, Poisson generalized linear model for count data with log-link, number of herbivore associations ~ number of locations found, McFadden's pseudo $R^2 = 0.27$, glm effect estimate = 0.161, intercept = 0.866, $p = 2 \times 10^{-16}$), both with and without taking plant relatedness into account (n = 76, Poisson phylogenetic generalized linear model, number of plant associations ~ number of locations found, glm effect estimate = 0.156, intercept = 1.237, p (Wald's) = 0). For Heliconiines (top), the effect was even stronger, explaining about 80% of the variance (n = 51, Poisson generalized linear model for count data with log-link, number of plant associations ~ number of locations found, McFadden's pseudo $R^2 = 0.775$, glm effect estimate = 0.152, intercept = 0.964, $p = 2 \times 10^{-16}$), with a similar effect size both with and without taking insect relatedness into account (n = 45, Poisson phylogenetic generalized linear model, number of plant associations ~ number of

locations found, glm effect estimate = 0.101, intercept = 2.09, p (Wald's) = 0).

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VITA

Marie Rose Clifford was born to Edwin and Alice Clifford in Iwakuni, Japan, and raised in Anacortes, Washington, U.S.A. She graduated from Anacortes High School and went on to obtain a Bachelor of Science in Biology, with a minor in chemistry, at the University of Washington in Seattle. After a gap year working with children in the foster care system, Marie returned to the University of Washington to pursue graduate work. During her time UW, Marie won a NSF Graduate Research Fellowship; co-founded and co-directed a citizen science and outreach initiative, the Urban Pollination Project, with fellow Biology graduate student Susan Waters; and worked full-time as a data scientist in industry starting in 2015. She completed her Ph.D. in Biology in May of 2017.