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Synchrony, function, and diversification of floral scent in Hawaiian *Schiedea* (Caryophyllaceae)

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

in Ecology and Evolutionary Biology

by

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DEDICATION

To

my parents

for their unending love and support

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ABSTRACT OF THE DISSERTATION

Synchrony, function, and diversification of floral scent in Hawaiian *Schiedea* (Caryophyllaceae)

by

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Doctor of Philosophy in Ecology and Evolutionary Biology

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Floral scent is a complex form of chemical communication between plants and pollinators that may be a part of the evolution of plant reproduction. I studied temporal, geographic, and taxonomic variation in the floral scents of species and hybrids of *Schiedea* (Caryophyllaceae), an endemic Hawaiian genus with diverse pollination modes. To measure genetic variation in the composition and timing of floral volatile emissions, I used dynamic headspace sampling in a common environment followed by gas chromatography – mass spectrometry or proton transfer reaction – mass spectrometry.

The individual regulation of discrete volatiles allows plants to produce scent blends with multiple functions throughout the day and night. Two sympatric species from different clades in this group, *Schiedea kaalae* and *S. hookeri*, were hypothesized to have similar scents that attract their shared moth pollinator (*Pseudoschrankia brevipalpis*), but instead they produce qualitatively distinct scents that diverge at night and are dominated by volatiles from different

chemical classes. However, the daily timings of these scent emissions are similar, and in *S. kaalae* volatiles that are known to attract moths peak within the period of moth visitation in the field, while others peak during the day. The evening scent of *S. kaalae* flowers is sufficient to attract moths against a background of scent and visual cues from two wind-pollinated relatives from the same island, demonstrating how moths respond to species-specific scent cues.

If scent plays a role in pollinator attraction, it can maintain or erode species boundaries that contribute to adaptive radiations. Floral scent production could affect the ability of hybrids to attract pollinators and contribute to gene flow between species. Artificially produced reciprocal hybrids between these two sympatric *Schiedea* species have mostly intermediate scent phenotypes that combine the sets of volatiles produced by each species, at similar total rates of emission, so scent might not represent a postzygotic barrier.

Recent evolution of separate sexes from hermaphroditism and wind pollination from biotic pollination in *Schiedea* may affect the functions of floral scent, and emissions of attractive compounds may be lost. In *S. globosa*, a subdioecious species distributed over multiple islands, scent composition varies over the course of the day, between sexes, and across reproductively isolated populations, and interpopulation scent divergence increases with genetic distance in males. The nightly increase in a set of volatiles was unexpected for a wind-pollinated species, especially because this set was not shared with two moth-pollinated relatives. This work highlights the differences in floral scent in related species that have evolved similar and distinct modes of pollination, describes the genetic variation in scent among isolated populations that may lead to scent divergence at longer evolutionary timescales, and suggests that some but not all temporal changes in floral scent are related to pollination.

INTRODUCTION

Many flowering plants use floral features such as color, shape, and size to advertise their rewards of nectar, pollen, mating, and nursery sites to animals which results in effective pollination. In addition to visual displays, chemical displays composed of multiple discrete volatile organic compounds can also mediate interspecies communication (Steiger et al., 2010) and carry dynamic and detailed information (Raguso et al., 2008, Schiestl, 2015). Floral scents can enhance plant reproduction by signalling species identity, rewards, or defenses, attracting pollinators, and repelling antagonists (Baldwin et al., 1997, Theis and Adler, 2012, Kessler et al., 2013). As a result of their impact on fitness, floral scent traits may experience strong natural selection (Schiestl et al., 2011, Parachnowitsch et al., 2012, Ehrlén et al., 2012).

These selective forces and the genetic variation that they act on have led to diversification of these chemical signals at various taxonomic scales. At the smallest scale, a spatial mosaic of pollinators may cause geographically structured selection that drives divergence in floral scent composition among isolated plant populations (Friberg et al., 2013, Gross et al., 2016). Comparative studies of floral scent have highlighted the rapid changes in floral scent composition and regulation that occur with pollination shifts within a genus (Raguso and Pichersky, 1995) or across the angiosperms (Jürgens et al., 2013), and how they drive and maintain speciation (Okamoto et al., 2015). These examples show how floral scent evolution that begins as differences among populations can influence adaptive radiations. I studied how floral scent variation is structured in a radiation of the Hawaiian plant genus *Schiedea* (Caryophyllaceae). *Schiedea* taxa, many of which are critically endangered, have undergone multiple shifts in pollen vectors (moths, wind, and presumably birds) and breeding systems (e.g., hermaphroditism, dioecy, and autogamy; Wagner et al., 2005, Willyard et al., 2011). I examined

variation in floral scent across time periods, sexes, populations, species, and pollination modes to understand how scent signals have responded to these evolutionary changes.

The timing and amplitude of shifts in volatile emissions determine the signals that nocturnal or diurnal pollinators and antagonists receive (Majetic et al., 2009, Morinaga et al., 2009, Yon et al., 2017, Chapurlat et al., 2018, Fenske et. al 2018). Therefore, plants may temporally regulate scent in a way that maximizes pollen import and export and minimizes energetic and ecological costs of scent emission (Yon et al., 2017). These ecological costs may come in the form of herbivores or other antagonists attracted by floral scent (Theis and Adler, 2012, Schiestl 2015, Nunes et al., 2016). The predicted synchrony of individual volatile emissions and pollination has often been observed at coarse time scales (e.g., Okamoto et al., 2008), but not yet at fine time scales (< 1 h). In my first chapter (Powers et al., 2020), I studied the floral scent of two sympatric species of *Schiedea* (*S. kaalae* and *S. hookeri*) from different lineages that share an endemic pollinator (Weisenberger et al., 2014, Weller et al., 2017) and hypothesized that the fine-scale timing and composition of scent emissions would be similar to attract this pollinator. Previous field observations allowed me to test which volatiles were most synchronized with the period of pollinator activity, a novel approach that allows separation of potential attractive signals from the "noise" of compounds not present when pollinators are active. I found that despite sharing a pollinator, these species produced distinct scents that diverged in composition in the evening. However, the timing of evening peaks of the volatiles produced by each species was similar, and for *S. kaalae* fell within the period of pollinator activity. In my second chapter, I analyzed field experiments that demonstrate that the scent of *S. kaalae* is attractive to its moth pollinator in the absence of visual cues. Together, these results indicate that for sympatric species that share a pollinator, timing of volatile emissions may be more important than specific

attractants, and in this nocturnal pollination system moths can discriminate and follow species-specific floral scent.

By affecting attraction of similar or different pollinators, floral traits of two species can promote either outcrossing and gene flow, or reproductive isolation and speciation. Distinct floral scents have been shown to enforce reproductive isolation between closely related species through attraction of distinct pollinators (Bischoff et al., 2015, Gervasi et al., 2017). However, selection from the same pollinator has led to convergence in attractants across different lineages (Cortis et al., 2009, Gögler et al., 2009, Nunes et al., 2017). Differences in floral scents of species that share a pollinator may be limited to compounds that are not behaviorally active (Cortis et al., 2009), or limited to the set of compounds that a pollinator can detect and learn to associate with a reward. In addition to scent mediating prezygotic isolation between species, the scents produced by hybrids may lead to postzygotic isolation. If the floral scents of hybrids are attractive, pollinators moving between hybrids and parent species could lead to introgression (Svensson et al., 2016), but if novel scents are unattractive to parental pollinators they will contribute to the reproductive barrier between the species (Vereecken et al., 2010, Marques et al., 2016). In my second chapter, I examined the potential for the scent of hybrids between the two species to act as a postzygotic reproductive barrier by comparing their evening scents to those of the parent species. Reciprocal hybrids usually produced scent blends that combined volatiles from both parents at intermediate rates of emission, although a few hybrid individuals produced scents similar to one or the other parent species. Because scent emissions in hybrids were not reduced or composed of novel compounds, these scents could potentially be attractive to pollinators and enhance backcrossing and introgression, but confirmation of pollinator visitation to hybrids would require behavioral tests.

The loss of biotic pollination and reliance on wind dispersal of pollen can drive changes in floral scent due to relaxed selection for pollinator attraction. Evolutionary transitions to wind pollination are associated with reduction in floral volatile emissions (Welsford et al., 2016), or reduced antennal responses in potential pollinators (Wang et al., 2018). The reverse transition from wind to insect pollination has led to increases in scent production (Wang et al., 2018, Wragg and Johnson 2011). However, functions of floral scent unrelated to pollination, such as defense from pathogens, herbivores, or visitors that remove rewards without pollinating (Schiestl, 2010, Galen et al., 2011, Delle-Vedove et al., 2017) may remain or be enhanced in wind-pollinated species. For example, across a community, wind-pollinated species have lower emissions but not lower volatile richness (Farré-Armengol et al., 2015). While biotically-pollinated plants must balance attraction and deterrence of beneficial and antagonistic visitors (Kessler et al., 2013), wind-pollinated species are free from this constraint. Wind pollination is often associated with the evolution of separate sexes in flowering plants. Dioecious species may or may not evolve sexual dimorphism in floral scent, depending on their pollen vector, the genetic potential for dimorphism, and variation in selection acting on each sex (Ashman 2009). In *Schiedea*, wind pollination may enable the transition to separate sexes in habitats where pollinators are scarce (Weller and Sakai 1990). In my third chapter, I investigated the floral scent variation of a subdioecious wind-pollinated species, *S. globosa*, that has radiated across multiple islands. I quantified the changes in scent from day to night, between sexes, and across genetically isolated populations, and compared scent divergence with genetic and geographic distances. Surprisingly, many floral volatiles not found in two moth-pollinated relatives increase in emission at night, indicating this daily pattern is likely not vestigial. Scent composition differs between males and females and among populations, and divergence in scent increases with genetic divergence in males. Males produce more scent than females in the evening, which could result

from allometry in flower size. These results establish that wind-pollinated species can emit volatiles on a daily cycle (for an unknown reason), that scent variation among populations is related to isolation and genetic differentiation, and sexual dimorphism in scent can arise after the evolution of separate sexes from hermaphroditism in the absence of biotic pollination.

These studies of floral scent at multiple scales have shown that first, related species can emit different volatiles with similar timings to attract the same pollinator. Second, even in the absence of biotic pollination, volatiles can be regulated on a daily cycle and scent differences may accumulate in isolated populations. Third, the shifts from a hermaphroditic breeding system to separate sexes have allowed floral scent to diverge slightly between sexes in one species, perhaps due to changes in floral allometry. Overall, knowledge of the scent diversity within and between populations and species, with as yet unknown ecological consequences, provides further reasons for conserving genetic diversity in this genus. The demonstration of moth attraction by species-specific floral scent may have consequences for restoration efforts of both plants and pollinators, including the potential to assess pollinator presence with cut inflorescences. Further research could identify which volatiles are required for attraction, examine in a phylogenetic context how scent has evolved among breeding systems and pollination modes, and integrate floral scent variation with population genetics.

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CHAPTER 1: Floral scent composition and fine-scale timing in two moth-pollinated Hawaiian *Schiedea* (Caryophyllaceae)

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Abstract

Floral scent often intensifies during periods of pollinator activity, but the degree of this synchrony may vary among scent compounds depending on their function. Related plant species with the same pollinator may exhibit similar timing and composition of floral scent. We compared timing and composition of floral volatiles for two endemic Hawaiian plant species, *Schiedea kaalae* and *S. hookeri* (Caryophyllaceae). For *S. kaalae*, we also compared the daily timing of emission of floral volatiles to evening visits of their shared pollinator, an endemic Hawaiian moth (*Pseudoschrankia brevipalpis*; Erebidae). The identity and amount of floral volatiles were measured in the greenhouse during day and evening periods with dynamic headspace sampling and GC-MS (gas chromatography – mass spectrometry). The timing of emissions (daily rise, peak, and fall) was measured by sampling continuously for multiple days in a growth chamber with PTR-MS (proton transfer reaction mass spectrometry). Nearly all volatiles detected underwent strong daily cycles in emission. Timings of floral volatile emissions were similar for *S. kaalae* and *S. hookeri*, as expected for two species sharing the same pollinator. For *S. kaalae*, many volatiles known to attract moths, including several linalool oxides and 2-phenylacetaldehyde, peaked within 2 h of the peak visitation time of the moth which pollinates both species. Floral volatiles of both species that peaked in the evening were also emitted several hours before and after the brief window of pollinator activity. Few volatiles followed a daytime emission pattern, consistent with increased apparency to visitors only at night. The scent blends of the two species differed in their major components and were most distinct from each other in the evening. The qualitative difference in evening scent composition between the two *Schiedea* species may reflect their distinct evolutionary history and may indicate that the moth species uses several different floral cues to locate rewards.

Introduction

In flowering plants, attraction of pollinators is often required for reproduction, but the multimodal signals that attract pollinators are costly and require both carbon and energy (Dicke and Sabelis, 1989; Grison-Pigé et al*.,* 2001). Floral signals that attract pollinators may also attract visitors that reduce fitness such as herbivores (e.g. Theis and Adler, 2012; Schiestl 2015; Nunes et al*.,* 2016), nectar robbers (e.g. Kessler et al*.,* 2008; Kessler and Halitschke, 2009), or generalist pollinators with high heterospecific pollen loads (Morales and Traveset, 2008). Selection on floral signals via pollinators is therefore expected to favor allocation of resources to traits that optimize pollen received or dispersed and minimize costs of apparency to other visitors. When pollinators are active only during a specific time period, temporal regulation of a floral signal is one way to increase efficiency in signaling (Hoballah et al*.,* 2005). For example, the fitness of *Nicotiana attenuata* plants is affected if the timing of flower orientation or olfactory pollination cues is altered physically or genetically (Baldwin et al., 1997; Yon et al*.,* 2017). Overlap between the window of pollinator activity and the timing of floral signals is common, whether the signals are related to physical access (Overland, 1960; Goldblatt et al*.,* 2004), flower orientation (Yon et al*.,* 2017), or scent production (Heath et al*.,* 1992; Huber et al*.,* 2004; Effmert et al*.,* 2005; Kumano and Yamaoka 2006; Okamoto et al*.,* 2008; Prieto-Benítez et al*.,* 2016; Chapurlat et al*.,* 2018).

These and other previous studies have been useful in identifying the volatiles emitted during a known period of animal activity, for example during the foraging periods of diurnal versus nocturnal pollinators (Bischoff et al*.,* 2014). Knowledge of how closely the time courses of volatile emissions match the activity of a pollinator is still limited, especially since pollinator activity can also change on very short time scales (Herrera, 1990; Knop et al., 2017). Here we generate continuous measurements of volatile emissions to observe the start and end of emissions, so that we can determine if volatiles are emitted outside of the period of pollinator activity and thus at times when costs might exceed benefits for a channel of information for the pollinator. Continuous measurements can also distinguish a volatile that is rising in emission, which might indicate a period of pollinator activity is starting, from a volatile that is declining at a given point in time.

Plant species with the same pollinator might be expected to display similar floral signals, but

most tests of floral scent convergence within genera have been restricted to flowers that mimic a female insect (Cortis et al., 2009; Gögler et al., 2009) or oviposition site (Jürgens et al., 2013) or provide a fragrance reward (Nunes et al., 2017). These pollination systems require the presence of key compounds in precise ratios to produce a successful mimic or species-specific pheromone. Food-seeking pollinators may not require such highly specific floral chemical displays. Plant species that reward pollinators with food and have distinct scents might nevertheless attract shared pollinators if pollinators learn to associate the scent of each species with a reward. If heterospecific pollen transfer between related species reduces fitness (by clogging stigmas or producing infertile hybrids), plants would benefit from species-specific signals if distinct scents reduce heterospecific pollen transfer through floral constancy of pollinator individuals (Waelti et al., 2008).

We investigated the composition and timing of floral scent in *Schiedea kaalae* and *S. hookeri* (Caryophyllaceae), two hermaphroditic species with specialized floral nectaries and similar floral morphology (Wagner et al*.,* 2005 *b*) which are pollinated by the endemic Hawaiian moth *Pseudoschrankia brevipalpis* (Erebidae; Weisenberger et al*.,* 2014; Medeiros, 2015; Weller et al*.,* 2017). In this plant genus, wind pollination evolved from biotic pollination (Sakai et al*.,* 2006, Willyard et al., 2011). Reversals from wind to biotic pollination are also possible but cannot be currently verified given the poor resolution of the clade containing nearly all wind-pollinated species as well as several hermaphroditic species, including *S. hookeri* (Willyard et al., 2011). The clades containing *S. kaalae* and *S. hookeri* diverged c. 1.3 Mya (Willyard et al., 2011). Because these species share the same moth pollinator, which visits for a brief period of time in the early evening, we predicted that the two *Schiedea* species would share similar timing of maximum emissions of compounds known to attract moths, but differ in evening floral scent composition due to their separate evolutionary histories.

We first describe the patterns of volatile emissions in these two moth-pollinated species by asking how *S. kaalae* and *S. hookeri* differ in the composition (identity and amount) of evening floral volatile emissions. Next we characterize how individual volatiles change throughout the day and night in each species. Finally, we quantify the degree of overlap of volatiles (in aggregate and individually) with pollinator activity for one of the species, *S. kaalae*.

Materials and Methods

Study system

Schiedea kaalae Wawra (sect. *Mononeura*) and *S. hookeri* A. Gray (sect. *Schiedea*) are hermaphroditic, self-compatible, protandrous, perennial herbs native to O‘ahu, Hawai‘i, USA, where populations of the two species occur in sympatry in parts of the Wai‘anae Mountains (*S. kaalae* [410 - 730 m above sea level, asl] and *S. hookeri* [260 - 870 m asl], Wagner et al*.,* 2005 *b*) and can flower at the same time. *Schiedea kaalae* also occurs in the Ko‘olau Mountains (Wagner et al*.,* 2005 *b*). Both species are listed as endangered by the US Fish and Wildlife Service and critically endangered by the IUCN (Ellshoff et al*.,* 1991; Bruegmann and Caraway, 2003; Wagner et al*.,* 2005 *a,* Bruegmann et al., 2016), and a total of only about 28 *S. kaalae* individuals in five populations remained in the wild before restoration efforts (Weisenberger et al*.,* 2014), precluding studies of the remnant populations *in situ*. *Schiedea hookeri* is more common in nature than *S. kaalae*, and large populations also exist following restoration efforts (D. Sailer, personal communication). The species produce inflorescences with 20-300 (*S. kaalae*) or 20-150 (*S. hookeri*) flowers per inflorescence and both species possess similar floral morphology with reflexed sepals 3-4 mm long, no petals, 10 stamens, 3 styles, and 5 nectaries (Wagner et al*.,* 2005 *b*).

Prior studies of pollination biology

The shared moth pollinator of *Schiedea kaalae* and *S. hookeri, Pseudoschrankia brevipalpis* (Weller et al*.,* 2017), perches on flowers (or more rarely, on other parts of the inflorescence) and feeds on nectar extruded from the tips of specialized tubular nectary extensions adjacent to the stamens (Weisenberger et al*.,* 2014; Harris et al*.,* 2012). At ‘Ēkahanui Gulch (Wai‘anae Mountains) *P. brevipalpis* was the only visitor to flowers of *S. kaalae*, based on observations over three years (Weller et al., 2017). Fewer pollinator observations were made for *S. hookeri* because of the inaccessibility of the sites, although direct and indirect observations both indicated that *P. brevipalpis* was the primary pollinator at ‘Ēkahanui Gulch (Weller et al., 2017). Very low numbers of other endemic moth species were observed visiting *S. hookeri* at a second site and a few carried *Schiedea* pollen, but pollen deposition was threefold lower than at ‘Ēkahanui Gulch, and no correlation between moth scales and pollen deposition was observed, indicating the absence of effective pollination (Weller et al., 2017). No daytime floral visitors to

either species have been observed (Weisenberger et al., 2014).

The elliptic flight patterns of the moths before they land on flowers suggest they rely little on visual targeting even before dark and are characteristic of moths seeking floral volatiles through anemotaxis (upwind flight; Cardé and Willis, 2008; Weller et al., 2017 and videos therein).

New analyses of field data for time of moth visits

For comparison with timing of volatile emissions, we determined the timing of flower visits by the moth *P. brevipalpis.* Our earlier studies (Weller et al., 2017) reported the duration of visits to flowers in male and female stages of anthesis but not arrival times. Here we analyzed arrival time of visits (landings on a flower) of *P. brevipalpis* to *S. kaalae* at ʻĒkahanui Gulch (*n* = 48 visits on three consecutive dates in March 2014 and one in July 2014; landings occurred from 17:49 - 19:28 HAST, 0.2 - 1.6 h after sunset). Observations of the field population always began at least a half hour in advance of any moth visit and continued until after moth activity ceased, so the entire spectrum of potential arrival times was included. We did not include *S. hookeri* in the analysis of timing of visits because we had too few direct observations of pollinator visits.

Because the timing of moth behavior and floral volatile emission patterns may be driven by light levels (Altenburger and Matile, 1990; Hansted et al., 1994; Hendel-Rahmanim et al., 2007) or circadian rhythms entrained by light cycles (Kolosova et al., 2001; Fenske et al., 2015; Yon et al., 2016, Fenske et al., 2018), we calculated the difference between the times of each moth visit to a flower and local sunset. The angle of elevation to the nearby ridge towards the median solar azimuth at sunset across observation dates was used to determine local sunset, using the *crepuscule* function of the R package *maptools* (Bivand and Lewin-Koh, 2019). This technique corrects for the shadows cast by the mountainous terrain. We combined these relative times across dates to create a temporal distribution of moth visits to *S. kaalae*.

Plants sampled

Volatile emissions were measured on plants of *Schiedea kaalae* and *S. hookeri* grown in the University of California, Irvine greenhouse. Plants were potted in UC mix (a soil mix developed by the University of California; 1:1:1 sand, peat, and redwood fiber) with added perlite and watered as needed with dilute liquid fertilizer (Grow More; 20-20-20 NPK plus micronutrients). Plants were grown from seeds or cuttings of six populations from the Waiʻanae Mountains (10

S. kaalae and 10 *S. hookeri* plants, Supplementary Table 1.S1; all collections were made before species were listed as federally endangered in 1991 and 1996, for *S. kaalae* and *S. hookeri,* respectively). Plants also were grown from intraspecific (mostly interpopulation) crosses between cultivated plants from these populations (22 *S. kaalae* plants, 22 *S. hookeri* plants). Interpopulation crosses within species were used because most natural populations now consist of a single individual and are highly inbred (Weisenberger et al*.,* 2014). For GC-MS measures, we sampled 32 plants of each species in the evening (see below). Four *Schiedea kaalae* and eight *S. hookeri* plants from this group were also sampled during the day. For continuous PTR-MS measurements of plants in a growth chamber over multiple days, we sampled five *S. kaalae* plants, two from Pu‘umaialau (Takeuchi 3587) and three from Pahole Gulch (Weller and Sakai 904), and three *S. hookeri* plants, one from Kalua‘a Gulch (Weller and Sakai 879, 400 m south of Pu‘uhapapa) and two from Wai‘anae Kai (Supplementary Table 1.S1). All plants chosen had ≥ 10 open flowers. The numbers of open male- and female-phase flowers, closed (post-anthesis) flowers, and floral buds were recorded immediately after sampling for both methods. Inflorescence age, as estimated by the ratio of closed to open flowers, did not vary between species in the sampled plants (ANOVA, P = 0.90, n = 64).

Scent collections and analysis by GC-MS

Scent collections

Procedures for dynamic headspace sampling for GC-MS were modified from Campbell et al. (2019). Scent traps, consisting of a glass capillary tube filled with 5 mg of Tenax TA and held with plugs of silanized quartz wool, were cleaned before initial use by heating in helium carrier gas for 5 min at 250 °C. Scent samples were collected from November 2016 - April 2017 in the greenhouse during evening and daytime sampling periods. The natural day length varied from 10 - 12 h. For the evening period, samples were taken with pumping start times between 16:30 - 21:00 PST (2.5 h before sunset - 3.9 h after sunset, mean ± SD relative to sunset 1.4 ± 1.3 h, with 86 % of samples taken after sunset). This wide sampling window was used to capture the potential gradient along the transition from light to dark, which was treated as a linear rather than discrete effect in the analysis (see below). For the day period, samples were taken from the same inflorescence earlier in the same day (start times 12:50 - 13:50 PST, 0.8 - 2.0 h after solar noon). Each plant was used on one date only. Dynamic headspace samples of floral volatiles were taken by enclosing inflorescences in 19 x 10 cm nylon-6 oven bags (Reynolds,

USA). Volatiles were allowed to equilibrate for 30 min at 22 - 32 °C (day) or 20 - 26 °C (evening) and pumped for 30 min through a scent trap using a pump (Supelco PAS-500, Spectrex, Redwood City, California, USA) set to a pre-trap flow rate of 200 mL/min. Ambient controls (*n* = 19) were taken from an empty oven bag sampled for the same duration to identify contaminants (see below). Samples were stored in capped glass vials at -20 °C until analysis.

GC-MS analysis

Floral scent composition (the identity and emission rate of each volatile in the overall scent blend) was characterized and quantified by thermal desorption gas chromatography-mass spectrometry (TD-GC-MS). We employed an Agilent 6890N GC (Agilent Technologies, Palo Alto, California, USA), with a 30 m × 0.25 mm internal diameter x 0.25 μm film thickness HP-5ms column (Agilent). The flow of helium carrier gas was 1 mL/min. Scent traps were placed in the sample tube of a Markes UNITY 2 thermal desorption device, purged with helium for 1 min, heated to 200°C for 5 min while re-trapping on Tenax adsorbent at 25 °C, and desorbed at 200°C for 3 min. After a 2 min hold at 40 °C, the temperature of the GC oven was ramped to 210 °C at 10 °C/min, then to 275 °C at 30 °C/min and held for 2 min. A coupled Agilent 5973N MSD mass spectrometer was operated in electron-impact ionization mode at 70 eV and scanned in the range 50-500 *m/z* at 3 s^{-1}.

Peak deconvolution, integration, and tentative compound identification were performed in the Automated Mass Spectral Deconvolution and Identification System (AMDIS) using the NIST 2017 mass spectral library. Components were included if they had mass spectral match scores greater than 75%, had maximum abundances across samples greater than 120,000 counts (6.6% of the median sample), and occurred in more than one sample. After calibration with a C_7-C_{30} alkane ladder, compound identities were verified by comparing retention indices (RI) with those given in the NIST library. Volatile emission rates were calculated within each compound class from peak integrations by calibration across 4 orders of magnitude with 7 authentic standards ((Z)-hex-3-en-1-ol, α-pinene, indole, linalool, β-caryophyllene, benzaldehyde dimethyl acetate, (E,E)-farnesol) in hexane applied to scent traps. Compounds in floral samples that did not exceed the amounts in ambient controls or GC blanks were considered contaminants (using t-tests with alpha adjusted by the false discovery rate method) and excluded from analyses. Based on the PTR-MS data, oct-1-en-3-ol and (Z)-hex-3-en-1-ol were likely induced by handling the inflorescences because both sharply decreased in the first two hours after bagging. Both

compounds can be induced by mechanical damage (Ozawa et al*.,* 2000; Leitner et al*.,* 2005; Kigathi et al*.,* 2009; Boggia et al*.,* 2015). We excluded (Z)-hex-3-en-1-ol from GC-MS analyses because its emissions remained low for days after the initial bagging, but because oct-1-en-3-ol resurged consistently at night (Supplementary Figure 1.S2), likely indicating floral emission, we included it in analyses. Emission rates were standardized by the number of open flowers.

Statistical analyses of scent composition

The total scent emissions per flower during the evening sampling period were compared between species with a Mann-Whitney test. To identify volatiles that differed between the two species and between times of day, we employed canonical analysis of principal coordinates (CAP; Anderson and Willis, 2003; Campbell et al*.,* 2016) with Bray-Curtis dissimilarities, as implemented in the function *capscale* from the R package *vegan* (R Core Team 2018; Oksanen et al*.,* 2018). This constrained ordination method is suited to discover multivariate patterns among predefined predictors, in this case, species, time relative to sunset (as a continuous variable because sampling windows were wide), and their interaction. We used a permutation test (*anova.cca*) to test each term of the full model sequentially and determine whether there was a significant interaction after accounting for the main effects. For visualization and to improve interpretation of the time axis, CAP was repeated within each species with time of day as the constraining variable. The CAP method constructs metric multidimensional scaling (MDS) axes to summarize variation that is not explained by the predictors. Volatile emission rates were square-root transformed to reduce skew before analysis.

Scent analysis in real time by PTR-MS

Advantages of real-time sampling

To identify temporal patterns of scent emissions and pollinator activity, most studies have compared scent (all volatiles and their emission rates) and pollinator activity during two discrete daily sampling periods (e.g. Prieto-Benítez et al*.,* 2015). More intensive sampling has yielded qualitative comparisons between selected scent compounds at 1 h resolution and pollinator visitation rates in three daily periods (Dötterl et al., 2012 *b*), and between overall scent intensity at 10 min resolution and a time range of pollinator visits (Dötterl et al., 2012 *a*). To make fine-scale comparisons that quantify scent-pollinator overlap, we take advantage of proton

transfer reaction mass spectrometry (PTR-MS) to generate continuous measurements of volatile emissions for multiple days, rather than the average emissions across a sampling period generated by trapping followed by GC-MS. We then compare those time courses with information on timing of pollinator visits at the scale of quarter hours using an overlap statistic to quantify the degree of synchrony. Prior studies with PTR-MS have revealed the daily emission profiles of individual volatiles (Abel et al*.,* 2009), and overlap between thermogenesis and scent signals (Marotz-Clausen et al*.,* 2018), but have not previously been paired with fine scale information on timing of pollinator visits.

Proton transfer reaction time-of-flight mass spectrometry (PTR-MS) allows extremely sensitive, real time quantitation of plant volatile emissions by using hydronium ions for chemical ionization (Lindinger et al*.,* 1998; Jordan et al*.,* 2009). Through direct ionization of the sample gas, PTR-MS can measure small molecules that are not efficiently trapped on adsorbents. Identification of individual components of complex mixtures with PTR-MS is difficult due to fragmentation and overlap of ions at unit mass resolution, but the technique has been used successfully on complex biological samples when paired with GC-MS to positively identify the volatiles expected in the mixture (Eugenio et al*.,* 2007; Cappellin et al*.,* 2012; Masi et al*.,* 2015; Schuhfried et al*.,* 2017). Identifications can be made for ions not found in the GC-MS spectra from standards reported in the literature.

PTR-MS experiment

Emission rates of volatiles are often highly sensitive to the environment (Farré-Armengol et al., 2014; Burkle and Runyon 2016; Campbell et al., 2019) and thus could differ between the growth chamber and field sites where the moths were studied. To minimize these variations, we sampled floral volatiles for 2 - 4 days with PTR-MS under environmental conditions similar to the sites where pollinator observations were conducted. Unlike emission rates, timings of volatile emissions are known to be driven by either direct light cues or the circadian clock calibrated by light cues, and are not expected to differ relative to those light cues (Fenske et al., 2018). We lined up temporal patterns of volatiles to those that occur under field conditions by expressing time courses relative to the time of sunset or the light-to-dark transition in the photochamber and using light conditions (intensity and photoperiod) and temperature conditions typical of the field. The remaining differences between the field and the photochamber were that temperature was kept constant to observe changes in emission rates not driven directly by heating, and the light

transitions were abrupt rather than gradual so that volatiles that respond to light could be distinguished from those with slower regulation.. The detailed methods for sampling, data processing, verification with reference standards, and identification are reported in Supplementary Methods S1.

Statistical analysis of volatile time courses

To visualize patterns of multivariate change in scent through time and between the species, we performed a principal components analysis of the PTR-MS ion time series with maxima over 0.001 counts·s^{-1}·$flower^{-1}$ for all plants at all time points (van Ruth and de Visser, 2015). All ions that met this criterion were analyzed, including those not identified by GC-MS or comparison to reference spectra.Time points were connected with lines to show the progression of each plant through scent space over multiple days. To identify volatiles with similar patterns of emission over time, we constructed WPGMA hierarchical clusterings of Pearson distances (Liao, 2005) among ion time series (with each ion signal scaled to its maximum). The resulting clustering of volatiles, visualized in a clustered heatmap, reflects similarity in both temporal patterns and presence or absence in each species.

To model the temporal peaks of individual volatile emissions, we fit Weibull functions to each ion time series for each plant and each day using the R package *cardidates* (Rolinski et al*.,* 2007). These functions allow different slopes in the rising and falling periods, and different baseline levels before and after the peak. From these fits we extracted the times of the beginning of exponential increase (0.5% of the modelled peak area), maximum, and end of exponential decrease (99.5% of the modelled peak area). For each species, we calculated the median time of maxima for each ion across days and plants.

To quantify the degree of scent-pollinator synchrony in *S. kaalae*, we compared the 24-hr distributions of *P. brevipalpis* visits across all dates to both a) the modelled times of maximum emission (from the fitted Weibull function) aggregated across all PTR-MS ions, days, and plants (which provides a single metric of synchronization between pollination and the timing of peaks across all scent compounds) and b) the actual time courses of emissions for each PTR-MS ion across days and plants (which shows which volatiles are the most or least synchronized with pollination). After aligning the sunset time to the dark transition in the growth chamber, we placed times of moth visits into bins that were 16 min in duration, centered on the 4-min

sampling blocks for each plant. We normalized each distribution to have an area of one, and then calculated the areal overlap between the two distributions (defined as the integral of the minimum of the two distributions; Miller-Rushing et al*.,* 2010). This statistic is affected by the position of the two distributions relative to each other, and the match in their width. We define the null expectation as the overlap between the moth visit distribution and a flat line, where the flat line represents either a) a uniform distribution of times of maxima or b) a hypothetical volatile holding a constant emission rate throughout the day.

Compounds attractive to moths

Selection for overlap between emission of a specific compound and moth visitation might be more likely if the compound is one that moths respond to behaviorally. The behavioral responses of *Pseudoschrankia brevipalpis* to individual floral volatiles are unknown, so we surveyed the literature for information on the detectability (search terms: moth + {antenna, EAD, EAG}) and attractiveness (search terms: moth + {attraction, behavior}) of the volatiles produced by *Schiedea* inflorescences. Electroantennographic detection (EAD) studies were used to determine whether a compound can be detected by moth antennae. Evidence of moth attraction is presented from behavioral tests. In these studies, the volatile was considered attractive if it induced more interactions than the control. Volatiles were applied to either an open trap with a scent emitter, a scent emitter within a wind tunnel, or a flower spiked with additional scent. From the literature, we recorded the number of moth species, their families, and the apparatus used (Supplementary Table 1.S2).

Results

Species differences in floral scent

Using GC-MS we detected 32 floral volatiles produced by *S. kaalae* and 36 produced by *S. hookeri*, for a total of 40 volatiles present in > 20% of samples of either species. These included 19 aliphatics, 7 benzenoids, 5 irregular terpenes, and 9 monoterpenes (Table 1.1). Of the 40 compounds, 28 were produced by both species. The literature survey of electrophysiological and behavioral studies in other moth species showed 9 are EAD-active (with no behavioral data available), 12 are EAD-active and attractive, one is not attractive, and no data are available for the others (Supplementary Table 1.S2). Including rarer volatiles and excluding two putative

wound volatiles, a total of 74 volatiles were detected and used for analysis.

Schiedea kaalae produced more total scent per flower than *S. hookeri* in the evening in the GC-MS measurements (median ± median absolute deviation 23 ± 12 ng·flower^{-1}·h^{-1} compared to 5.0 ± 3.9 ng·flower^{-1}·h^{-1} for *S. hookeri*, Mann-Whitney test, $U = 179$, $P < 10^{-10}$). Major components of the scent blends differed (CAP species effect, Table 1.2a). For *S. kaalae* in the evening, three cyclic linalool oxides (the pyranoid oxide ketone, pyranoid oxide, and furanoid oxide) made up 67% of the average scent blend, followed by five volatiles each making up more than 1.5% of the blend: oct-1-en-3-ol, hexanal, octan-3-one, α-phellandrene, and 2-phenylacetaldehyde (Table 1.1, Fig. 1.1a). The evening blend was more complex for *S. hookeri* than *S. kaalae* (Shannon diversity index of 2.1 ± 0.3 [mean ± SD] versus 1.6 ± 0.2 for *S. kaalae*), and composed of 41%), followed by 11 volatiles each making up 1.5 - 10% of the blend: hexanal, octan-3-one, heptane-2,3-dione, cyclopentane-1,2-dione, two hexenal isomers, benzaldehyde, an unknown benzenoid, furan-3-ylmethanol, 3,5,5-trimethylhex-2-ene, and indole (Table 1.1, Fig. 1.1b). The first CAP axis that separated the floral scents of the species reflects these major differences (Table 1.2b).

These differences in evening scent between the two species were supported by PTR-MS measurements (Fig. 1.2). The two species produced distinct scent blends at all times of day (principal components analysis of ions in the PTR-MS spectrum across all timepoints, Fig. 1.3). The scent compositions of the two species were most distinct from each other during the evening (Fig. 1.3) and this was verified by the full CAP analysis of GC-MS volatile compositions (Table 1.2a, ordination not shown). Individuals from the two *S. kaalae* populations differed from each other in their evening scent composition (Fig. 1.3), primarily by the emission of indole by the two plants from Pu‘umaialau (Takeuchi 3587) which was absent in the three plants from Pahole Gulch (Weller & Sakai 904; both in the Wai‘anae range, Fig. 1.2, Supplementary Table 1.S1).

Daily patterns in floral scent

Comparisons between day and night using GC-MS

In both *Schiedea kaalae* and *S. hookeri*, total floral scent emissions increased and scent composition changed markedly in the evening. Median evening scent emissions measured by GC-MS for *S. kaalae* were 1.5 times higher than daytime emissions and 1.8 times higher for *S.*

hookeri. Scent composition varied by species, time of day, and their interaction (full canonical analysis of principal coordinates, Table 1.2a). The scent composition of individual plants changed between the day and evening within both species (time effects in separate CAP analyses: $F_{1,38}$ = 6.17, P = 0.0001 for *S. hookeri* and $F_{1,34}$ = 3.11, P = 0.0024 for *S. kaalae*). For *S. kaalae*, the volatiles with the highest evening loadings were linalool oxide (pyranoid), linalool oxide (furanoid), and 2-phenylacetaldehyde (Fig. 1.1a), all of which are EAD-active in moths (Supplementary Table 1.S2). In *S. hookeri*, volatiles with the highest evening loadings were the unknown benzenoid, 1-3-dihydro-2-benzofuran, and indole (Fig. 1.1b; indole attracts hawkmoths, (Supplementary Table 1.S2).

Fine scale timing using PTR-MS

The floral scents of both species intensified in the evening in the PTR-MS measurements (Supplementary Table 1.S3, Fig. 1.4) as they did with GC-MS. This daily modulation was driven by pulses of individual volatiles from diverse biochemical pathways with periodicity of approximately 24 h (Supplementary Fig. 1.S2). Each volatile had a distinctly-shaped time course (Fig. 1.2) but the times of maximum emission among the evening volatiles fell within a 4 h period (Fig. 1.4). The volatile emission patterns formed three main groups based on their starting times relative to the light and dark transitions (Supplementary Table 1.S3). Morning volatiles, such as acetaldehyde (m/z 45), started to rise from their baseline emission rates when plants are exposed to light, plateaued near their maximum within 1 h, began to fall at dark, and returned to baseline 1-5 h after dark. Afternoon volatiles, such as linalool ketone (pyranoid) (m/z 169), rose 0 - 6 h before dark, peaked 0 - 2.5 h after dark, and returned to baseline 4-10 h after dark. Some of the afternoon volatiles that started rising slowly in the afternoon showed an inflection point at the dark transition and began rising more quickly (Fig. 1.2, e.g. indole). Dark volatiles, such as benzaldehyde (m/z 107), rose at dark, peaked 1 - 3 h after dark, and returned to baseline 3-8 h after dark.

Both species started emitting more volatiles in the afternoon or after dark than in the morning (Supplementary Table 1.S3). Production of all of the known moth attractants started in the afternoon or after dark (Fig. 1.4). Daytime emission rates for many evening-peaking volatiles were generally very low, on the order of tens to hundreds of times less than emission rates in the evening (Fig. 1.4, Supplementary Table 1.S3), although some daily changes were more subtle (e.g. linalool oxide (pyranoid) ketone in *S. kaalae*; Fig. 1.2). The magnitude of the diel

ratio (the emission rate 2 - 3 h after dark relative to the rate 5-6 h before dark) varied between species for the same volatile (Fig. 1.4, Supplementary Table 1.S3); for example, *S. hookeri* showed more extreme increases at night than *S. kaalae* in methyl 2-aminobenzoate, indole, and the unknown nitrogen aromatic and unknown benzenoid. The temporal patterns were consistent across days, plants, and in some cases between species, although the volatile emissions of *S. hookeri* often started and peaked later compared to the same compound in *S. kaalae* (Fig. 1.4). In some plants, maximum emissions of some volatiles varied over consecutive days and generally decreased over time, perhaps due to aging of the inflorescence (Fig. 1.2, Supplementary Fig. 1.S2).

Overlap with moth visitation

Pseudoschrankia brevipalpis visited *S. kaalae* in ʻĒkahanui Gulch from 0.2 - 1.6 h after sunset (mean ± SD 1.1 ± 0.4 h after sunset, n = 48). For *S. kaalae*, most volatiles began emission 1 - 5 h before the first *P. brevipalpis* visit to any flowers, peaked 1.5 h before - 1 h after the mean time of moth visits, and returned to baseline 1 - 4 h after the last visit (times relative to sunset or the dark transition in the growth chamber, Fig. 1.4).

The areal overlap between the time of moth visitation to *S. kaalae* and the times of maxima across PTR-MS ions, days, and plants was 49%, much greater than the null expectation of 14% for a uniform distribution of maxima, given these moth observations. The median time of ion maxima was 1.6 h after dark for *S. kaalae* and 2.4 h after dark for *S. hookeri*. The time courses of individual *S. kaalae* volatiles varied in their degree of overlap with moth visitation (Fig. 1.4), with an unknown cyclohexane, the linalool oxides, 2-phenylacetaldehyde, and methyl 2-aminobenzoate having the highest overlap (both 2-phenylacetaldehyde and methyl 2-aminobenzoate are moth attractants; Supplementary Table 1.S2). The mean overlap for the individual time courses of *S. kaalae* volatiles and moth visits was 25 ± 16%, 25 ± 15% for EAD-active volatiles, and 30 ± 16% for moth attractants that were EAD-active (mean ± SD), compared to a null expectation of 14% overlap for volatiles emitted at a constant rate. Volatiles that rose in the morning and peaked during the day (such as acetaldehyde) had low overlap with moth visitation, and of this group only the green leaf volatile (Z)-hex-3-en-1-ol (m/z 101, PTR-MS ion signal shared with hexanal) was an attractant, for moths that feed on leaves (Supplementary Table 1.S2). The degree of overlap also varied across nights and plants, driven primarily by variation in the diel ratio and secondarily by changes in the timing of the maximum

(Fig. 1.4).

Discussion

In two *Schiedea* species pollinated by the same moth, the timing of emission of floral volatiles was more similar than the identity of the major compounds released by those species in the evening. The floral scents produced by *S. kaalae* and *S. hookeri* were noTable 1.for the biochemical diversity of compounds that oscillate between day and night. The timings of peak pollinator activity for *S. kaalae* and of peak emissions of known moth attractants was similar, although volatile emissions started prior to pollinator activity and continued after cessation of pollinator activity.

Moth attractants

Many volatiles that peak in the evening in *S. kaalae* and *S. hookeri* are typical benzenoid, oxygenated terpene, and nitrogen-containing floral attractants of crepuscular noctuid and sphingid moths (Supplementary Table 1.S2), such as those found in the nocturnal floral emissions of moth-pollinated orchids (Kaiser, 1993), *Nicotiana* (Loughrin et al*.,* 1991), *Petunia* (Hoballah et al*.,* 2005), and other diverse taxa (Knudsen and Tollsten, 1993; Dobson et al*.,* 1997; Miyake et al*.,* 1998). In other studies, the hawkmoth *Hyles lineata* shows antennal responses to many volatiles emitted in the evening by the two *Schiedea* species (Supplementary Table 1.S2).

The potential attractive role of these nocturnally-emitted compounds in *Schiedea* is highlighted by their increase in production with evolutionary transitions to moth pollination in several other genera. In *Clarkia*, production of linalool and linalool oxides (the pyranoid and furanoid forms produced by *S. kaalae*) evolved in a transition from bee to nocturnal moth pollination (Raguso and Pichersky, 1995). In *Ipomopsis*, indole (in our study produced primarily by *S. hookeri*) attracts hawkmoths to *I. tenuituba* but is not emitted by its hummingbird-pollinated sister species *I. aggregata* (Bischoff et al*.,* 2015). *Nicotiana bonariensis* produces the apocarotenoid 4-oxoisophorone and its variant 2,2,6-trimethylcyclohexane-1,4-dione (both produced by *S. kaalae*) from flowers that open at dusk and are pollinated by small crepuscular moths (Noctuidae) rather than the hawkmoths and hummingbirds attracted to close relatives of *N. bonariensis* that lack these compounds (Raguso et al*.,* 2003; Clarkson et al*.,* 2004; Kaczorowski et al*.,* 2005). None of the evening-peaking volatiles in *Schiedea hookeri* and *S. kaalae* were

present in the wind-pollinated *Schiedea* species (*S. globosa* and *S. kealiae*, Jürgens et al*.,* 2012) that *P. brevipalpis* largely avoided in field choice tests (Weller et al*.,* 2017).

Species differences in floral scent

In this study, *S. kaalae* and *S. hookeri* share a sole pollinator in an area of sympatry, but have different evolutionary histories, leading us to predict distinct floral volatile compositions. In sympatric species from different lineages of sexually-deceptive and oil-secreting orchids, similar selection pressures imposed by the same pollinator have driven convergence in overall floral scent, or in the subset of compounds that have antennal activity (Cortis et al., 2009; Gögler et al., 2009; Nunes et al., 2017). We found instead that the evening floral scents of the two *Schiedea* species pollinated by *P. brevipalpis* differ qualitatively in composition. Scent differences between the species are more accentuated during the evening than during the day, echoing the same pattern found in nine *Nicotiana* species, some of which are nocturnally pollinated by hawkmoths (Raguso et al*.,* 2003). The overall composition and major compounds of each species are unique: *Schiedea kaalae* produces a set of three linalool oxides and 2-phenylacetaldehyde, which are produced in relatively minute amounts by *S. hookeri,* and *S. hookeri* uniquely produces an unknown benzenoid and heptane-2,3-dione (Table 1.1). These qualitative differences could result from the evolutionary history of *S. hookeri*, which is in a clade of wind-pollinated species (*Schiedea* sect. *Schiedea*) and may represent a reversal to moth pollination from ancestral wind pollination (the current phylogenetic hypothesis does not fully resolve the direction of this shift, Willyard et al., 2011). However, both *S. kaalae* and *S. hookeri* produce the moth attractant benzaldehyde (Hoballah et al*.,* 2005) and the insect attractant oct-1-en-3-ol (Hall et al*.,* 1984), and *S. hookeri* emits the moth attractants indole (Bischoff et al*.,* 2015) and methyl 2-aminobenzoate (Bisch-Knaden et al*.,* 2018) which are emitted at lower rates by *S. kaalae* (Fig. 1.2). Experiments that test moth preferences in the field at sites of both species (as in Bischoff et al*.,* 2015) are needed to elucidate whether one critical volatile, a blend of the shared volatiles, or other factors are important for attraction of pollinators. Given the observed differences in scent between these related species that share the same moth species as a pollinator, future community studies should not always assume strict similarity in scent composition across unrelated plant taxa visited by the same pollinator or pollinator guild. Instead, distinct sets of compounds may be perceived by those pollinators.

Overlap with moth visitation

Our work builds on diverse examples of synchrony in floral signals and pollinator activity during the day (Matile and Altenberger, 1988; Kite and Smith, 1997; Dötterl et al*.,* 2012 *a*; Nunes et al*.,* 2016) and night (e.g. Nilsson, 1983; Dötterl et al*.,* 2005; Hoballah et al*.,* 2005; Dötterl et al*.,* 2012 *b*; Steen et al*.,* 2019) and enhances temporal resolution to characterize the overlap of pollinator activity and floral volatile production. In both *Schiedea* species, the emissions of many floral volatiles were restricted to the afternoon and evening hours and in *S. kaalae* peaked within 2 h of the mean time of *P. brevipalpis* visits in the field (Fig. 1.4). In *S. kaalae*, the distribution of timings of maximum emissions across all volatiles, days, and plants indicated a good but imperfect temporal match between potential signals and the insect receiver. The volatiles that peak during the day and fall at dark would not be perceived by crepuscular moths after sunset, and their patterns of emission were all consistent with induction by light. The daytime volatiles could be related to photosynthesis (in the bracts of *S. hookeri*) or transpiration, rather than pollinator attraction (as is the case for both the daytime-peaking acetaldehyde and ethanol, its precursor; Graus et al., 2004). The maximum emissions of *S. hookeri* evening volatiles were shifted about 1 h later on average than their counterparts in *S. kaalae*, and many *S. hookeri* volatiles continued to be emitted until the early morning. These differences could stem from alternate temporal selection pressures (perhaps moths visit *S. hookeri* at a later time than they visit *S. kaalae*), or differences in evolutionary history of the plant species.

In *S. kaalae*, many volatile emissions spanned a much broader time range than the period of moth visitation. This could indicate constraints on how fast volatile emissions can be modulated, low ecological costs (e.g. apparency to herbivores) or low energetic costs of volatiles at those times, or a marginal benefit of attracting any moths that may be active at those times. Early initiation of volatile emission (i.e., for the volatiles that rose in the afternoon) could create a long downwind scent plume for long-distance attraction of moths (Supplementary Table 1.S3, Cardé and Willis, 2008). Conversely, the volatiles that rise after dark just as moths are beginning to forage could be important for short-distance attraction. The peaks of individual *S. kaalae* evening volatiles differed in their degree of overlap with the distribution of moth visitation (20 - 55%; Fig. 1.4). Known moth attractants, but not EAD-active volatiles, had slightly higher areal overlap in time with moth visits than the mean across all volatiles. This areal overlap statistic captured temporal differences from both early or late shifts in the time course of emissions and

differences in peak width (narrow or broad), the two types of differences that are characterized in studies of phenology (Miller-Rushing et al., 2010). These two components were also examined separately by calculating times of maxima and diel ratios. Either type of difference could affect how and when pollinators or other visitors could perceive these volatiles.

Daily regulation of attractants may increase the fitness of plants by reducing energetic costs, and it may also serve to reduce the attraction of plant antagonists that use the same floral cues as pollinators (Baldwin et al., 1997; Nunes et al., 2016). No native florivores or herbivores have been reported on outplanted or natural populations of these or any other *Schiedea* species. Though the fitness costs of emitting the evening volatiles during the day are unknown, the high level of daytime and before-dawn suppression indicates they could be substantial.

Floral scent is a complex trait in both synthesis and perception, and identification of volatiles or suites of volatiles that serve different functional roles (defense, attraction, metabolism) within diverse scent blends is challenging. However, categorizing volatiles by their pattern of temporal regulation (Nielsen et al., 1995; Marotz-Clausen et al., 2018) narrows the set of compounds that potentially influence the behavior of pollinators with constrained windows of activity. Follow-up behavioral studies might be able to test these candidate volatiles to confirm a function. In this case, volatiles could be classified by whether they increased immediately with light (e.g. monoterpenes), increased in the afternoon without a light cue (e.g. pyranoid linalool ketone), or increased after dark (e.g. benzaldehyde; Fig. 1.2, Fig. 1.4). Volatiles could also be ranked by their relative change in emission rate when the pollinator is active vs. not active, and by their overlap with pollinator visitation. Future studies could investigate the proximate causes of regulation of these volatiles (e.g. by the circadian clock, reviewed in Fenske and Imaizumi, 2016), and identify which class is most attractive to pollinators. We predict that the afternoon-rising volatiles are long-range attractants because they would diffuse a great distance by the time moths are active, allowing moths to detect the population. Volatiles that increase after dark may be short-range attractants because they would not establish a long scent plume by the time moths are active.

Conclusions

Almost all volatiles released from inflorescences of *Schiedea kaalae* and *S. hookeri* displayed

strongly time-specific modulations. Most *S. kaalae* volatiles peaked during or several hours after the brief time of evening visitation of *Pseudoschrankia brevipalpis*, a pollinator of both species. This pattern is generally consistent with selection that maximizes the attraction of pollinators by producing volatiles when pollinators are active, but the emission of most evening volatiles extended hours before the period of pollinator activity, when they could be active in long-range attraction. Additionally, some volatiles, perhaps unrelated to pollinator attraction, followed a daytime cycle. The composition of volatiles differed markedly between species, especially in the evening, and yet the timings of peak emissions were similar between the species. Knowing when emissions of each volatile begin, peak, and end will help to focus studies on the ecological functions of volatile compounds based on their temporal overlap with the activity of mutualists and antagonists.

Supplementary Material

Supplementary Material includes Table 1.S1: Localities of *Schiedea* populations in this study, Table 1.S2: Evidence of EAD (electroantennographic detection) responses or attraction of moths for compounds detected by GC-MS, Table 1.S3: Diel changes in floral scent measured by PTR-MS, Methods S1: PTR-MS methods, Figure 1.S1: PTR-MS spectra of reference standards, Figure 1.S2: Heatmap of volatile emissions measured by PTR-MS.

Data Availability Statement

The datasets generated for this study are available in Dryad at https://doi.org/10.7280/D12H4M.

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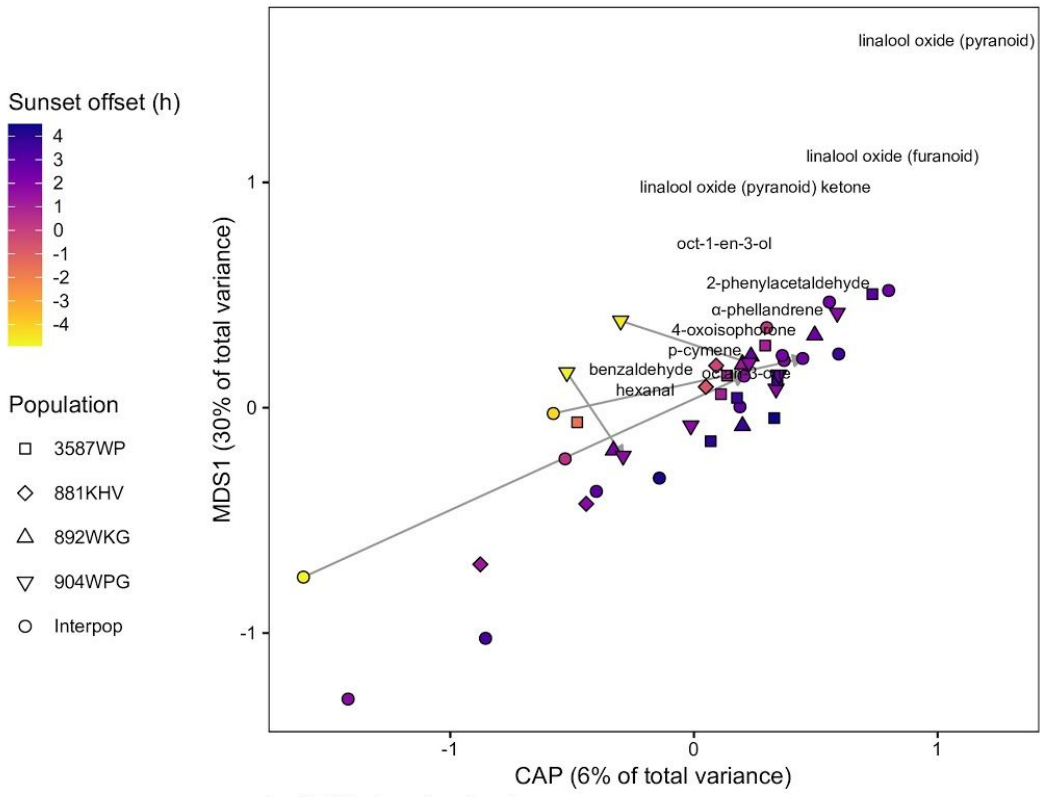
Figures

Figure 1.1

Floral scent composition determined by GC-MS of (a) *Schiedea kaalae* and (b) *S. hookeri* plants sampled at different times of day, visualized by canonical analyses of principal coordinates (CAP). The CAP axis shows scent variation explained by time of day (evening on the right), and the first multidimensional scaling (MDS) axis shows additional unconstrained scent variation. The shape of the points indicates the source population number (collection locations in Supplementary Table 1.S1) or a plant from a cross between populations ('Interpop'). Color indicates the time of collection, with zero indicating sunset, positive values indicating time after sunset, and negative values indicating time before sunset. Arrows connect samples of the same inflorescence during the day and following night. The names of volatiles are positioned by their CAP and MDS scores and labelled if they are > 0.05 units from the origin.

a. Schiedea kaalae



b. Schiedea hookeri

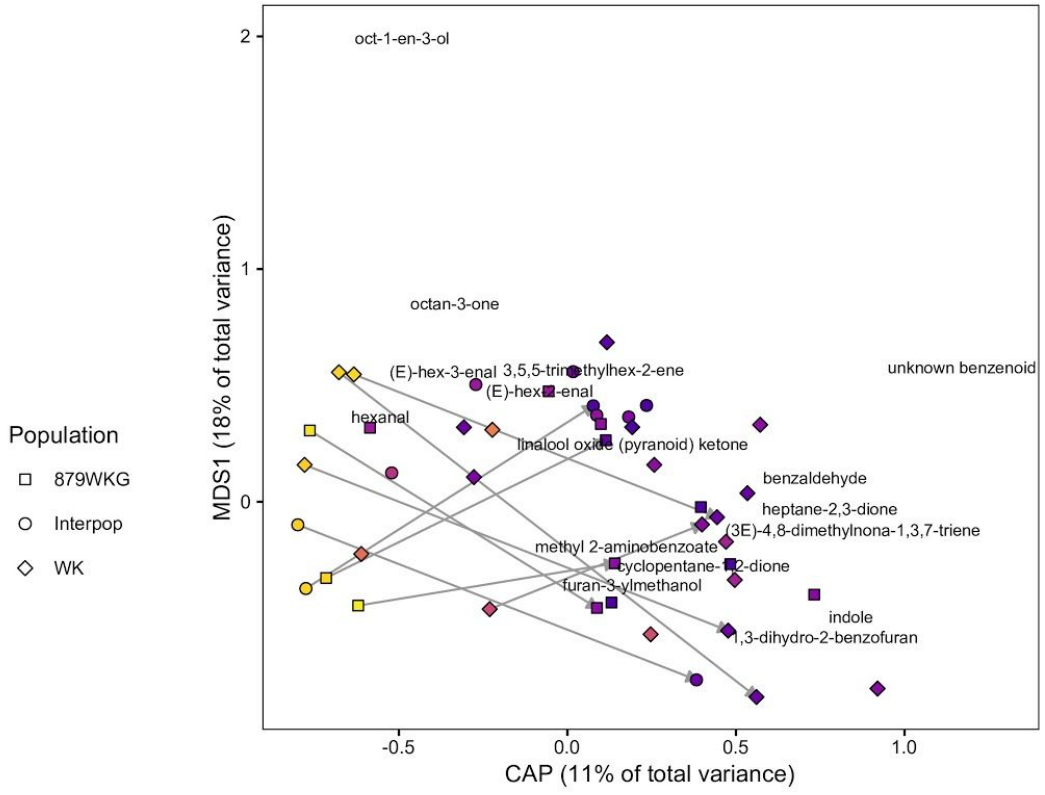


Figure 1.2

PTR-MS signals per flower (arbitrary units) for floral volatile emissions from five *Schiedea kaalae* plants (green shades) and three *S. hookeri* plants (purple shades) across 2 - 4 d. Periods of darkness in the growth chamber are indicated by darker gray shading. Plants are named with their population number and a letter (collection locations in Supplementary Table 1.S1), and colored by population. Panels present those PTR-MS ion signals (*m/z* value given in the label) that correspond to molecular or fragment ions of volatiles identified by GC-MS in evening scent emissions. Scales vary according to the maximum signal per flower, displayed next to each panel.

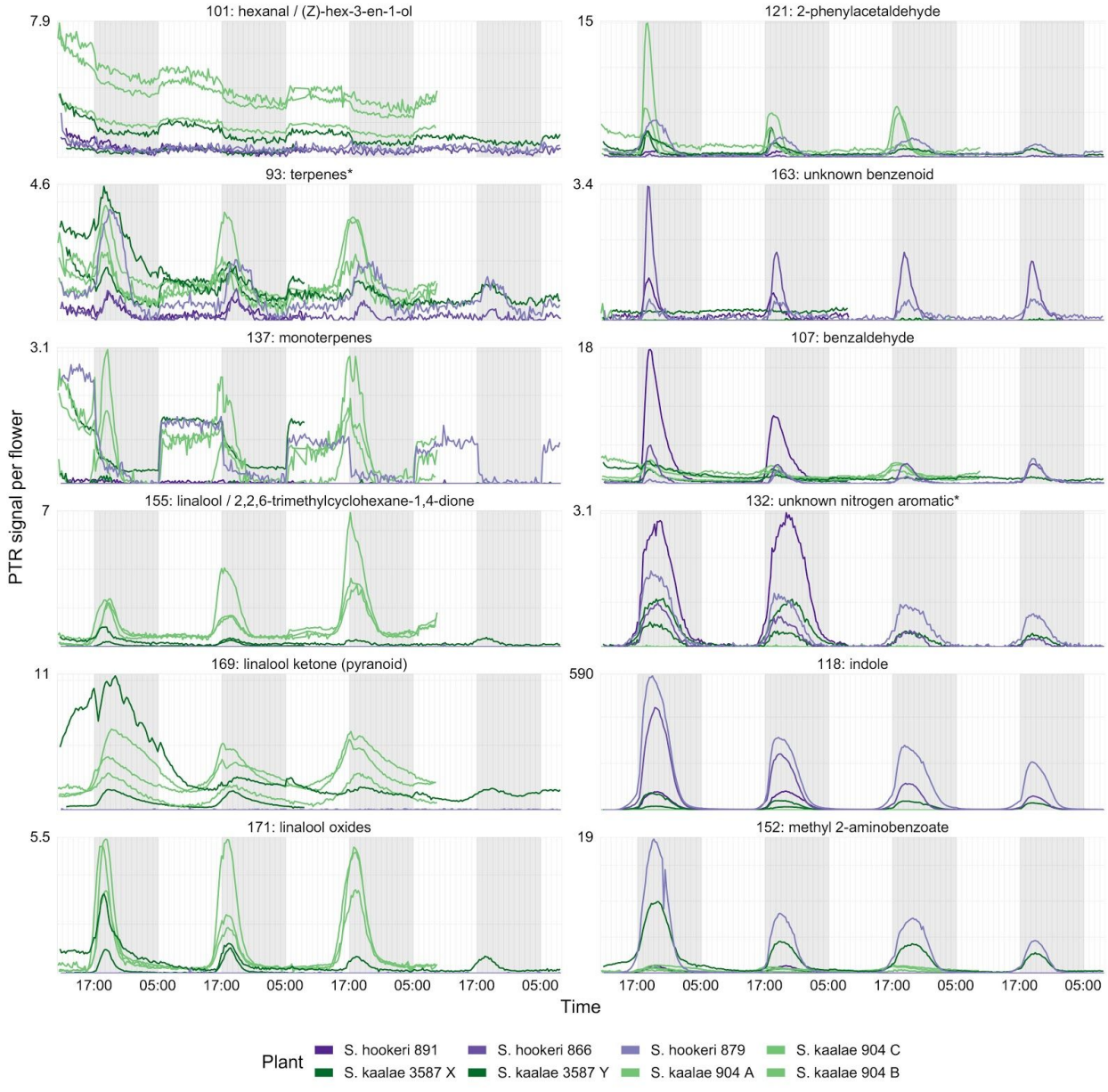


Figure 1.3

Daily patterns of floral scent in the same five *Schiedea kaalae* plants and three *S. hookeri* plants as in Fig. 1.2 mapped by principal components analysis (PCA) of PTR-MS ion signals with maxima over 0.001 counts·s^{-1}·$flower^{-1}$, including unidentified ions. The first and second principal components are shown on the vertical and horizontal axes. Loadings for each ion are indicated by the black *m/z numbers* (Supplementary Table 1.S3). Lines connect adjacent time points for each plant. Time of day is represented by different colors on the line, with transitions from dark to light at 5:00 (cyan-green) and light to dark at 17:00 (orange-red) marked on the scale. The source population (904, 3587, 879, WK) is indicated by the shape of the points (collection locations in Supplementary Table 1.S1). Each plant was sampled for 2 - 4 d.

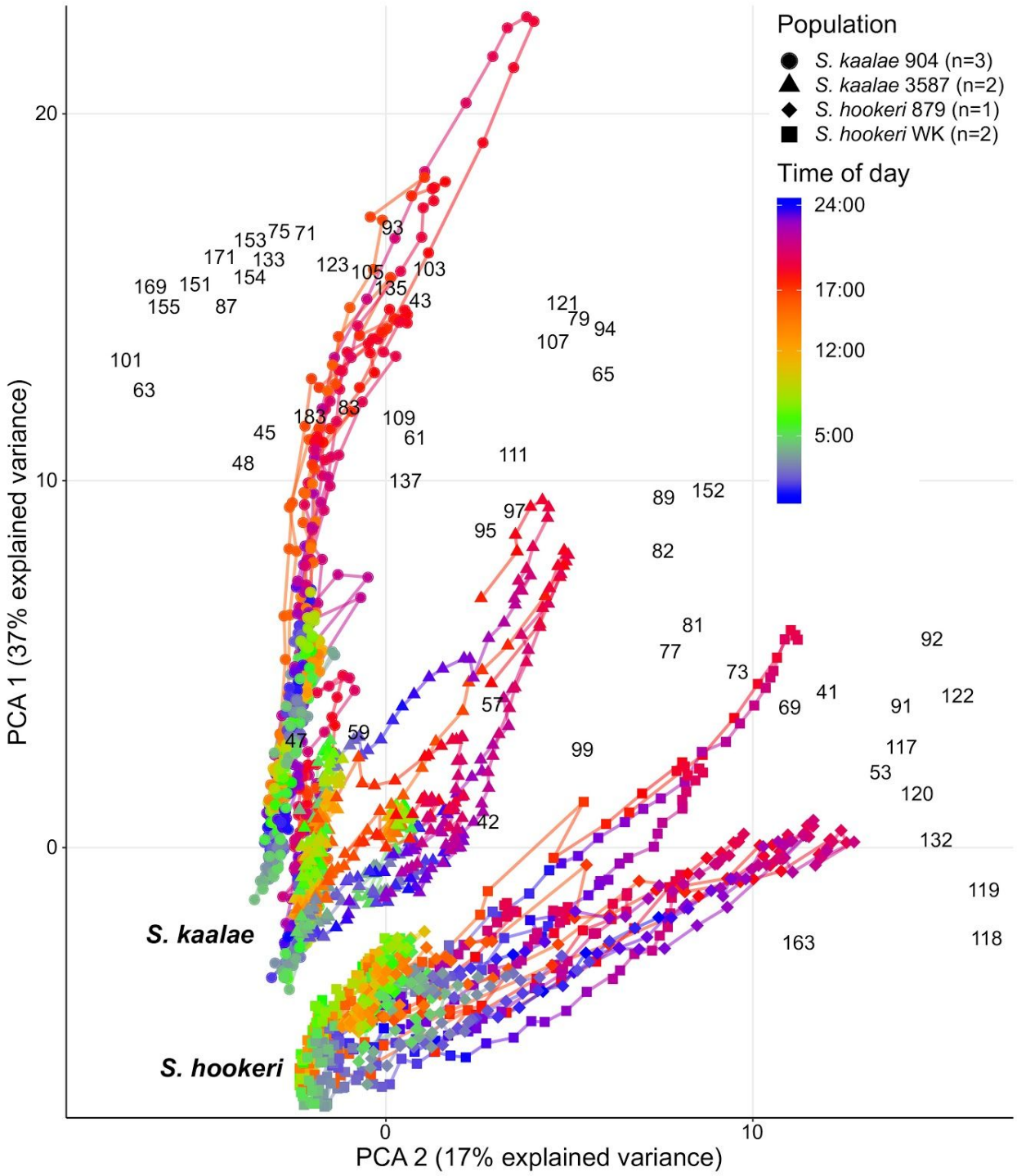

Population
S. kaalae 904 (n=3)
S. kaalae 3587 (n=2)
S. hookeri 879 (n=1)
S. hookeri WK (n=2)
Time of day
24:00
17:00
12:00
5:00
PCA 1 (37% explained variance)
PCA 2 (17% explained variance)
20
10
0
0
10
S. kaalae
S. hookeri

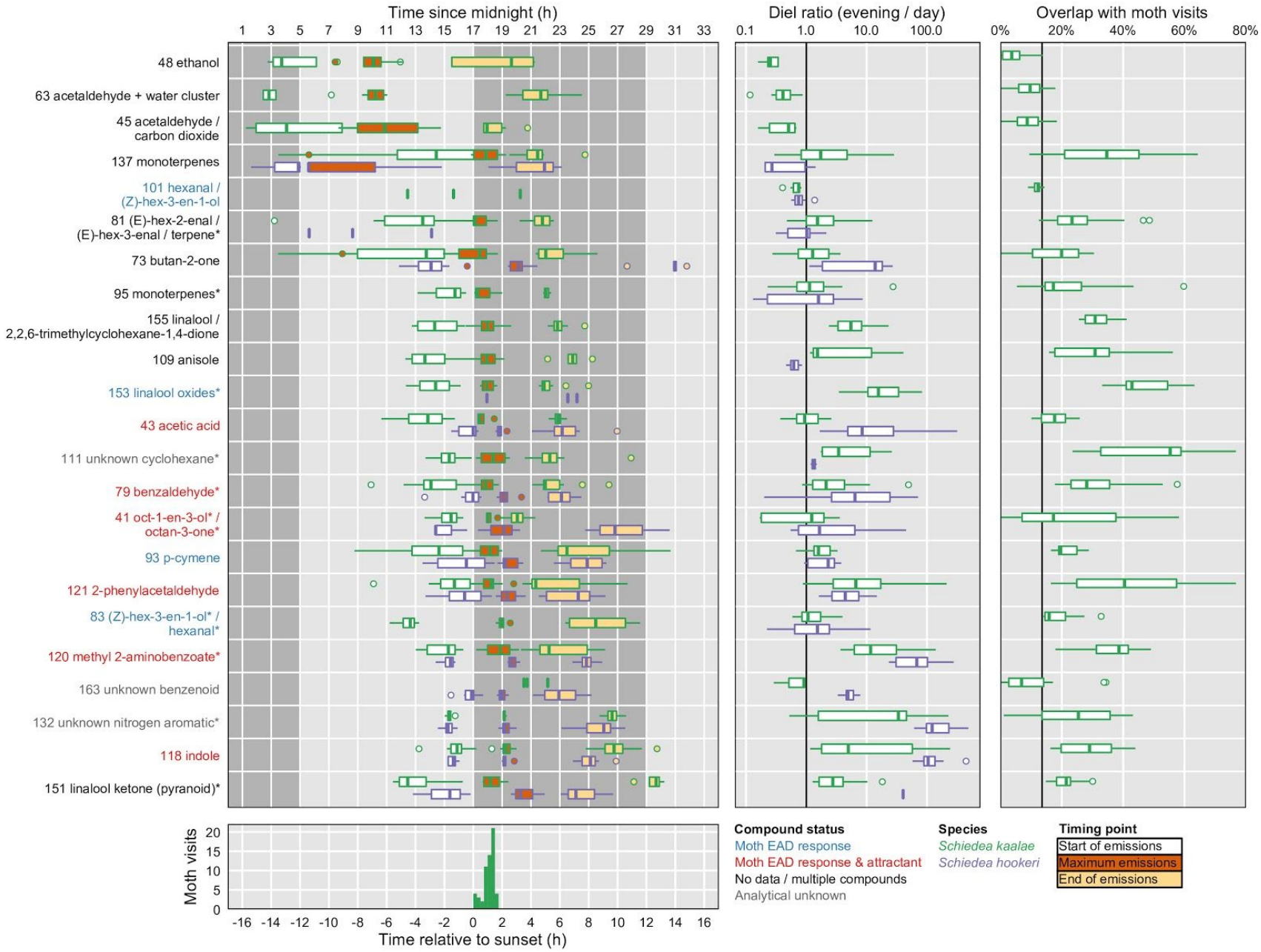
Figure 1.4

Moth foraging activity and summaries of temporal peaks of floral volatile emissions of *Schiedea kaalae* (green) and *S. hookeri* (purple). Boxplots contain the median, first and third quartiles, range, and outliers (beyond 1.5 times the interquartile range from the first or third quartile). *Left:* The timing of volatiles emissions. Tentative identifications for each ion are given after their protonated *m/z* value. Fragment ions are indicated by an asterisk by the name. One PTR-MS ion per compound is shown (Supplementary Table 1.S3), for ions with maxima over 0.001 counts·s^{-1}·$flower^{-1}$ for all plants at all time points. For each ion and species, three boxplots summarize (across all plants and days) the start (white), maximum (maroon), and end (light orange) of emissions. Timing points were inferred by fitting Weibull functions to ion signals and trimming to 99% of the fitted peak area. Ions are arranged vertically by the mean starting time relative to the dark transition in the growth chamber. Light and dark periods in the growth chamber are indicated by background shading. The dark transition in the growth chamber was approximately coincident with the ambient greenhouse sunset time. Ion labels are colored by whether they elicit a moth antennal EAD response (blue), elicit an EAD response and attraction (red), are not reported in the literature (black, labelled 'no data'), contain signals from multiple compounds (black), or are analytical unknowns (gray, all references in (Supplementary Table 1.S2) except acetic acid, Knight et al. 2011).

Middle: The magnitude of daily changes in emission of floral volatiles. Boxplots show the diel ratio in emissions (evening/day) for evening (19:00 - 20:00 PST, 2 - 3 h after dark) and day (12:00 - 13:00 PST, 5 - 4 h before dark) for each plant and date. A ratio > 1 (right of vertical bar) indicates that emissions increased in the evening.

Right: The overlap of *S. kaalae* volatile emissions with moth activity. Boxplots show the areal overlap value between two curves: the time course of each volatile relative to dark, and the distribution of *P. brevipalpis* visit times relative to sunset. Overlap values vary among plants and days. The vertical bar indicates the null overlap expectation for a hypothetical volatile that does not change in emission over the course of a 24 h period (14% overlap).

Bottom: Visits of *Pseudoschrankia brevipalpis* to *S. kaalae* flowers relative to sunset at the field site over four dates.


Time since midnight (h)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33
Diel ratio (evening / day)
0.1 1.0 10.0 100.0
Overlap with moth visits
0% 20% 40% 60% 80%
48 ethanol
63 acetaldehyde + water cluster
45 acetaldehyde / carbon dioxide
137 monoterpenes
101 hexanal / (Z)-hex-3-en-1-ol
81 (E)-hex-2-enal / (E)-hex-3-enal / terpene*
73 butan-2-one
95 monoterpenes*
155 linalool / 2,2,6-trimethylcyclohexane-1,4-dione
109 anisole
153 linalool oxides*
43 acetic acid
111 unknown cyclohexane*
79 benzaldehyde*
41 oct-1-en-3-ol* / octan-3-one*
93 p-cymene
121 2-phenylacetaldehyde
83 (Z)-hex-3-en-1-ol* / hexanal*
120 methyl 2-aminobenzoate*
163 unknown benzenoid
132 unknown nitrogen aromatic*
118 indole
151 linalool ketone (pyranoid)*
Moth visits
0 5 10 15 20
-16 -14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14 16
Time relative to sunset (h)
Compound status
Moth EAD response
Moth EAD response & attractant
No data / multiple compounds
Analytical unknown
Species
Schiedea kaalae
Schiedea hookeri
Timing point
Start of emissions
Maximum emissions
End of emissions

Supplementary Methods S1

Each PTR-MS sampling session lasted two to four days and involved three plants. For each of the three sessions, three flowering plants were selected from the greenhouse, watered in the morning, and placed in a growth chamber (the UCI Fluxtron) at 23 °C and 60% relative humidity. This temperature is similar to the mean monthly temperature (mean ± SD from 1968 - 1976: 24 ± 2 °C) at a weather station 7 km away and 100 m lower than the ʻĒkahanui Gulch site where pollinator observations were conducted (weather station Lualualei TWR NL 803.1, 21.4833° N, 158.1333° W, 458 m asl, data from the Global Historical Climatology Network, Menne et al., 2012). Plants received about 450 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, measured at the top of the inflorescence, from LED lights (LumiGrow Pro, LumiGrow, Emeryville, California, USA). This light level matches sunfleck conditions in Hawaiian mesic forest understory (Pearcy 1983). The photoperiod was 12 h light : 12 h dark, with immediate light transitions at 05:00 and 17:00 PST. For comparison, day length on Oʻahu ranges from 11 to 13 h throughout the year. Because sampling occurred at different times of year, these fixed times in the growth chamber were offset 0 - 1.75 h from the ambient photoperiod in the greenhouse. Several flowering inflorescences from each plant were placed in nylon-6 oven bag enclosures (40.6 cm x 44.4 cm). A fourth, empty enclosure was used as a blank sample. Each enclosure received dry zero air at the bottom of the enclosure from a zero-air generator (total of 12 L/min of air distributed among the 4 enclosures). Air was sampled from the top of the enclosure and 7 m long, 0.4 cm ID PFA sampling lines were heated at 50 °C. The enclosure was sealed with a tie around the peduncle, input and sampling lines, and thermocouple. Relays switched gas flow to the PTR-MS between the four enclosures, sampling the output flow from each plant or blank enclosure every 16 min in 4-min sampling blocks. This rotating sampling approach provides higher throughput compared to systems that sample one plant at a time. Input and output flows from each enclosure were

monitored. An Ionicon PTR-Time-of-Flight-MS 1000 ultra (Ionicon Analytik, Innsbruck, Austria) was used to monitor masses from 33-205 *m/z* (mass/charge ratio) and the signal at each mass was averaged and recorded at 1 s intervals. The PTR-MS inlet flow was 140 standard cubic centimeters per minute, and the drift tube operated at 2.8 mbar, 60 °C, and 600 V.

We examined the fragmentation patterns of nine reference compounds to aid in identifying compounds in the PTR-MS spectra. Reference compounds were selected based on GC-MS results and commercial availability. We measured three terpenes (α-phellandrene, α-pinene, (−)-linalool), four aliphatic compounds (oct-1-en-3-ol, octan-3-one, hexanal, (Z)-hex-3-en-1-ol), and two benzenoids (benzaldehyde and indole). The fragmentation patterns for terpenes and aliphatics change with the energy level E/N (Maleknia *et al.,* 2007; Pang 2015; Kari *et al.*, 2018). For each compound, 100 µL liquid standard or c. 20 mg solid standard was added to a 2 mL GC vial, sealed, punctured with 1 cm of 1.5 mm ID teflon tubing, and placed in a 0.5 L glass jar. Zero air was pumped into the jar at 5 L/min and outflow split between an exhaust tube and the PTR-MS inlet. Spectra were averaged over 10 min after a 3 min equilibration period, and measurements alternated between standards and the empty jar washed in methanol to prevent carryover. If an ion's signal rose twofold over the baseline, it was included in the spectrum for that compound. Under the PTR-MS conditions described, all reference compounds except indole underwent some level of fragmentation (Supplementary Figure S1). The terpenes α-phellandrene, α-pinene, and (−)-linalool showed typical fragmentation patterns (Tani *et al.,* 2003; Maleknia *et al.,* 2007; Misztal *et al.,* 2012; Tani 2013), each showing a major fragment ion at *m/z* 81. The aliphatics oct-1-en-3-ol, octan-3-one, hexanal, and (Z)-hex-3-en-1-ol and benzaldehyde also showed typical fragmentation (Buhr *et al.,* 2002; Maleknia *et al.,* 2007; Tasin *et al.,* 2012; Pang 2015).

Raw PTR-MS data and flow rates were processed using PTR-MS Viewer 3 (Ionicon Analytik, Innsbruck, Austria) and then in Igor Pro 7 (Wavemetrics, Inc., Lake Oswego, Oregon, USA) to calculate volatile fluxes from the difference between signals in the blank and plant enclosures. For analysis we rounded the *m/z* (mass-to-charge ratio) to integer values due to resolution limits of the instrument. Volatile fluxes were standardized to a per flower measure by dividing by the number of open flowers. We did not attempt quantitative calibration of volatile fluxes. Daily post-illumination bursts (on the order of minutes) of oct-1-en-3-ol and green leaf volatiles (C_6 alcohols and alkenes) were observed in all *S. hookeri* inflorescences (unlike *S. kaalae*, *S. hookeri* inflorescences produce 1-12 mm long leaf-like bracts that could contribute to these emissions; Wagner et al*.,* 2005). Green leaf volatiles are known to be emitted transiently when photosynthesis is halted (Graus et al*.,* 2004), particularly during recovery from plant stress (Jud et al*.,* 2016), and so for analysis we excluded all data from the first 4-min sampling period following the light-to-dark transitions.

Tentative identities (molecular ions, fragment ions, or combinations of unresolved ions) of each *m/z* value were established by comparison to molecular weights of compounds detected by GC-MS in each species and time period and to published and experimental measurement of reference compounds (Supplementary Figure S1). Overlap of two compounds of a particular mass occurred for the six- and eight-carbon alcohols and aldehydes and for the terpenes, but many other compounds yielded ions with unique masses. Overlap of ions between different compounds occurred either due to isomeric product ions, or the inability of the PTR-MS instrument to distinguish similar masses. The overlap in molecular and fragment ions complicates PTR mass spectral analysis of mixtures (Pang 2015), and here we attempt only to distinguish compounds with identical or overlapping fragments when they produce a separate unique ion. In all other cases we indicate ambiguity in assignment with a slash between the

potential contributors.

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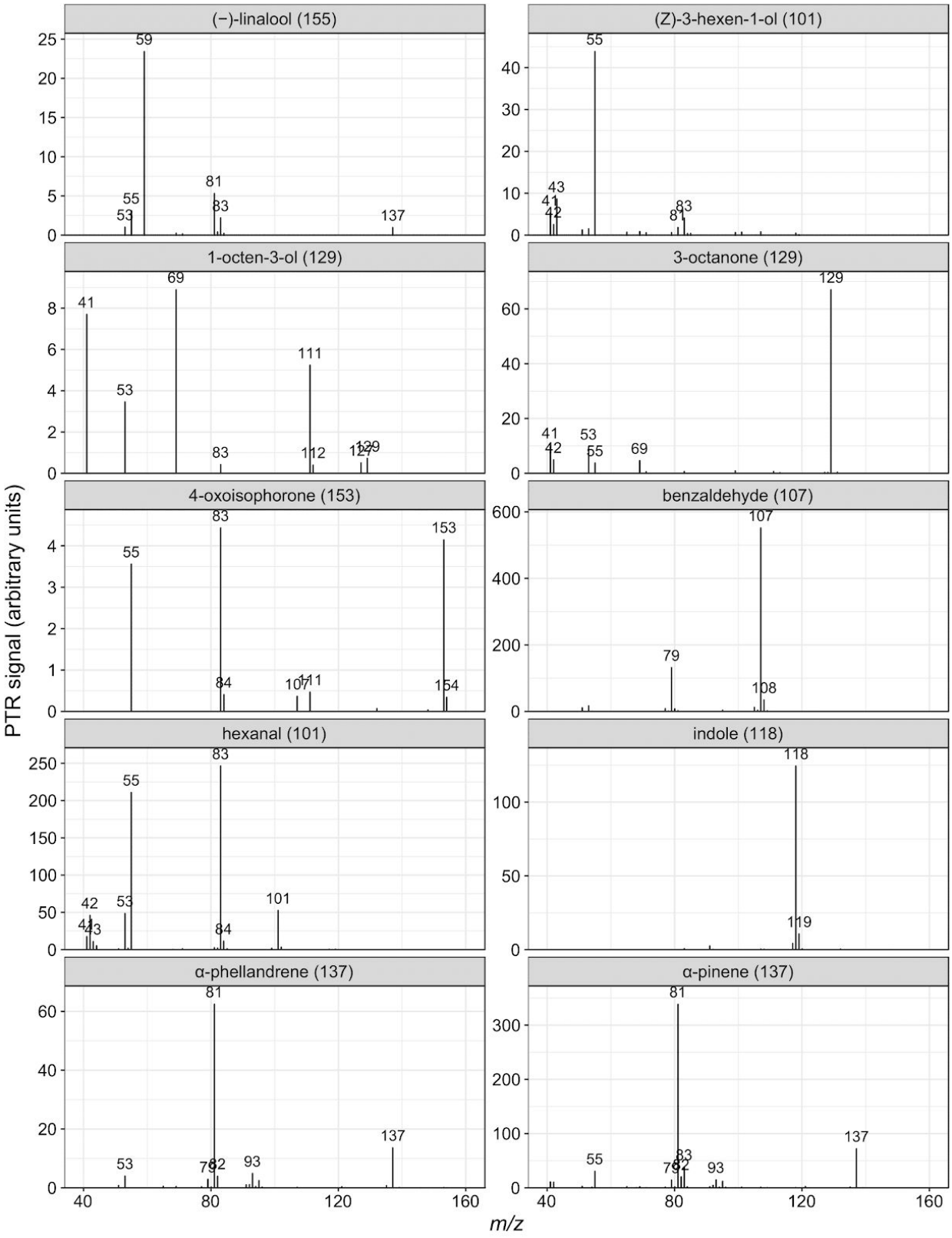
Supplementary Figure 1.S1

PTR-MS spectra of reference standards. Only ions with signals > 2 times the initial zero air reading are included. Scales vary between plots, as compounds with higher vapor pressures (Kim *et al.,* 2016) produced higher total filtered signals. The molecular ion M[H+] of each compound is indicated in parentheses.

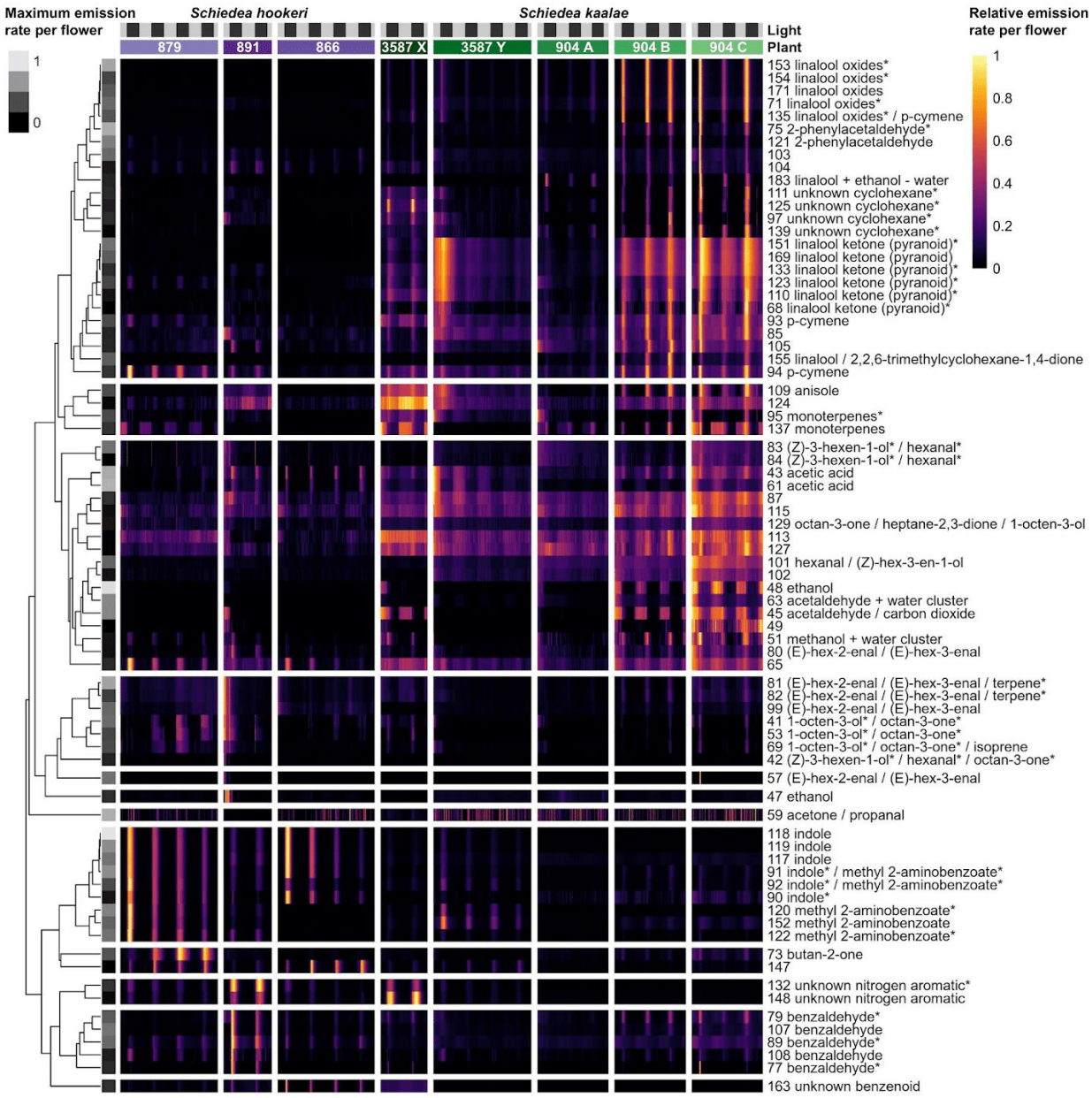
Reference

Kim, S., Thiessen, P. A., Bolton, E. E., Chen, J., Fu, G., Gindulyte, A., et al. (2016). PubChem Substance and Compound databases. *Nucleic Acids Res* 44, D1202–D1213. doi:10.1093/nar/gkv951.


(−)-linalool (155)
(Z)-3-hexen-1-ol (101)
1-octen-3-ol (129)
3-octanone (129)
4-oxoisophorone (153)
benzaldehyde (107)
hexanal (101)
indole (118)
α-phellandrene (137)
α-pinene (137)
PTR signal (arbitrary units)
m/z

Supplementary Figure 1.S2

Grouping of volatile emissions patterns for *Schiedea kaalae* (5 plants, green, right) and *S. hookeri* (3 plants, purple, left) measured by PTR-MS. Light/dark cycles in the growth chamber over 2-4 d for each of the 8 plants are indicated by dark and light bands (top row). Relative emission rates per flower are represented by colors from black to yellow (low to high, scale on the right) and are scaled by the maximum emission rate per flower of each ion. This maximum is indicated by colors from black to white (low to high) on the sidebar (left). Tentative identifications are given on the right for each ion (Table 1.3). On the left, ions are clustered by their scaled time series using WPGMA hierarchical clustering of Pearson distances. The clustering is separated into 11 groups by cutting across the dendrogram at a uniform height. Compounds are often represented by multiple fragment ions, indicated with asterisks, and a particular mass can show contributions from more than one compound or fragment, indicated by slashes.


Maximum emission rate per flower
1
0
Schiedea hookeri
Schiedea kaalae
Light
Plant
879
891
866
3587 X
3587 Y
904 A
904 B
904 C
Relative emission rate per flower
1
0.8
0.6
0.4
0.2
0
153 linalool oxides*
154 linalool oxides*
171 linalool oxides
71 linalool oxides*
135 linalool oxides* / p-cymene
75 2-phenylacetaldehyde*
121 2-phenylacetaldehyde
103
104
183 linalool + ethanol - water
111 unknown cyclohexane*
125 unknown cyclohexane*
97 unknown cyclohexane*
139 unknown cyclohexane*
151 linalool ketone (pyranoid)*
169 linalool ketone (pyranoid)
133 linalool ketone (pyranoid)*
123 linalool ketone (pyranoid)*
110 linalool ketone (pyranoid)*
68 linalool ketone (pyranoid)*
93 p-cymene
85
105
155 linalool / 2,2,6-trimethylcyclohexane-1,4-dione
94 p-cymene
109 anisole
124
95 monoterpenes*
137 monoterpenes
83 (Z)-3-hexen-1-ol* / hexanal*
84 (Z)-3-hexen-1-ol* / hexanal*
43 acetic acid
61 acetic acid
87
115
129 octan-3-one / heptane-2,3-dione / 1-octen-3-ol
113
127
101 hexanal / (Z)-hex-3-en-1-ol
102
48 ethanol
63 acetaldehyde + water cluster
45 acetaldehyde / carbon dioxide
49
51 methanol + water cluster
80 (E)-hex-2-enal / (E)-hex-3-enal
65
81 (E)-hex-2-enal / (E)-hex-3-enal / terpene*
82 (E)-hex-2-enal / (E)-hex-3-enal / terpene*
99 (E)-hex-2-enal / (E)-hex-3-enal
41 1-octen-3-ol* / octan-3-one*
53 1-octen-3-ol* / octan-3-one*
69 1-octen-3-ol* / octan-3-one* / isoprene
42 (Z)-3-hexen-1-ol* / hexanal* / octan-3-one*
57 (E)-hex-2-enal / (E)-hex-3-enal
47 ethanol
59 acetone / propanal
118 indole
119 indole
117 indole
91 indole* / methyl 2-aminobenzoate*
92 indole* / methyl 2-aminobenzoate*
90 indole*
120 methyl 2-aminobenzoate*
152 methyl 2-aminobenzoate
122 methyl 2-aminobenzoate*
73 butan-2-one
147
132 unknown nitrogen aromatic*
148 unknown nitrogen aromatic
79 benzaldehyde*
107 benzaldehyde
89 benzaldehyde*
108 benzaldehyde
77 benzaldehyde*
163 unknown benzenoid

Tables

Table 1.1

Evening floral volatile emissions from *Schiedea kaalae* and *S. hookeri* detected by GC-MS in > 20% of samples of either species (40 of 76 compounds, *n* = 32 plants for each species). Evidence of EAD (electroantennographic detection) responses or attraction of moths for these compounds is presented in Supplementary Table 1.S2.

					Proportion of evening samples [3]		Mean nonzero emission rate (ng/flower/hr)		Mean emission rate (ng/flower/hr)		Mean relative emission rate [4]	
Class	**RI [1]**	**Mean match score**	**CAS [2]**	**Name**	***S. kaalae***	***S. hookeri***	***S. kaalae***	***S. hookeri***	***S. kaalae***	***S. hookeri***	***S. kaalae***	***S. hookeri***
Aliphatic	796	87%	4440-65-7	(E)-hex-3-enal	0%	50%		0.56		0.28		3.4%
	797	94%	66-25-1	hexanal	**100%**	**100%**	0.56	0.80	0.56	0.80	3.4%	9.5%
	830	90%	96-04-8	heptane-2,3-dione	8%	**86%**	0.05	0.54	0.00	0.46	0.0%	5.6%
	840	91%	6728-26-3	(E)-hex-2-enal	0%	**81%**		0.43		0.35		4.2%
	848	94%	928-96-1	(Z)-hex-3-en-1-ol	21%	**89%**	0.24	6.64			excl.	excl.
	855	78%	7642-10-6	hept-3-ene	5%	22%	0.24	0.26	0.01	0.06	0.1%	0.7%
	855	84%	4412-91-3	furan-3-ylmethanol	8%	25%	0.10	0.95	0.01	0.24	0.0%	2.8%
	855	89%	2415-72-7	propylcyclopropane	32%	25%	0.08	0.33	0.03	0.08	0.2%	1.0%
	882	84%	2216-34-4	4-methyloctane	21%	3%	0.03	0.01	0.01	0.00	0.0%	0.0%
	901	92%	13129-23-2	methyl furan-3-carboxylate	0%	33%		0.03		0.01		0.1%
	905	92%	3008-40-0	cyclopentane-1,2-dione	21%	22%	0.78	1.85	0.16	0.41	1.0%	4.9%
	933	83%	18829-55-5	hept-2-enal	37%	0%	0.06		0.02		0.1%	
	949	79%	26456-76-8	3,5,5-trimethylhex-2-ene	5%	47%	0.05	0.40	0.00	0.19	0.0%	2.3%
	960	92%	3391-86-4	oct-1-en-3-ol	**100%**	**100%**	3.17	3.38	3.17	3.38	19.3%	40.6%
	961	92%	106-68-3	octan-3-one	**89%**	**97%**	0.49	0.72	0.44	0.70	2.7%	8.4%
	971	84%	111-13-7	octan-2-one	21%	0%	0.04		0.01		0.0%	
	981	92%	72237-36-6	hex-4-enyl acetate	0%	25%		0.35		0.09		1.1%
	1152	87%	53398-84-8	[(E)-hex-3-enyl] butanoate	0%	28%		0.29		0.08		1.0%
	1347	90%	31501-11-8	[(Z)-hex-3-enyl] hexanoate	0%	28%		0.27		0.07		0.9%
Benzenoid	896	91%	100-66-3	anisole	3%	39%	0.01	0.02	0.00	0.01	0.0%	0.1%
	937	95%	100-52-7	benzaldehyde	**100%**	**100%**	0.07	0.30	0.07	0.30	0.5%	3.7%
	1017	92%	122-78-1	2-phenylacetaldehyde	**95%**	50%	0.29	0.01	0.27	0.01	1.7%	0.1%
	1179			unknown benzenoid [6]	11%	**64%**	0.02	0.42	0.00	0.27	0.0%	3.2%

	1198	86%	103-70-8	N-phenylformamide	0%	25%		0.09		0.02		0.3%
	1268	92%	120-72-9	indole	13%	**89%**	0.02	0.16	0.00	0.15	0.0%	1.8%
	1316	94%	134-20-3	methyl 2-aminobenzoate	5%	**61%**	0.00	0.14	0.00	0.08	0.0%	1.0%
Irregular terpene	1086	82%	19945-61-0	(3E)-4,8-dimethylnona-1,3,7-triene	3%	**64%**	0.05	0.15	0.00	0.09	0.0%	1.1%
	1115	90%	1125-21-9	4-oxoisophorone	**100%**	3%	0.13	0.00	0.13	0.00	0.8%	0.0%
	1120	87%	28564-83-2	3,5-dihydroxy-6-methyl-2,3-dihydropyran-4-one	16%	22%	0.14	0.13	0.02	0.03	0.1%	0.3%
	1139	87%	20547-99-3	2,2,6-trimethylcyclohexane-1,4-dione	**84%**	0%	0.06		0.05		0.3%	
	1322	81%	141891-14-7	4-hydroxy-2,6,6-trimethyl-3-oxocyclohexene-1-carbaldehyde	**71%**	3%	0.04	0.04	0.03	0.00	0.2%	0.0%
Mono-terpene	964	84%	123-35-3	β-myrcene	47%	3%	0.02	0.02	0.01	0.00	0.1%	0.0%
	978	90%	99-83-2	α-phellandrene	**76%**	42%	0.46	0.04	0.35	0.02	2.2%	0.2%
	997	89%	99-87-6	p-cymene	**55%**	22%	0.12	0.06	0.07	0.01	0.4%	0.2%
	1013	85%	3779-61-1	(E)-β-ocimene	26%	0%	0.02		0.00		0.0%	
	1061	94%	5989-33-3	linalool oxide (furanoid)	**100%**	22%	2.13	0.08	2.13	0.02	13.0%	0.2%
	1071	82%	78-70-6	linalool	34%	47%	0.11	0.05	0.04	0.03	0.2%	0.3%
	1087	93%	33933-72-1	linalool oxide (pyranoid) ketone	**100%**	**75%**	4.82	0.11	4.82	0.08	29.4%	1.0%
	1152	93%	39028-58-5	linalool oxide (pyranoid)	**100%**	22%	3.98	0.10	3.98	0.02	24.2%	0.3%
	1245	78%	EPA-7965	epoxy-linaloloolo xide	39%	0%	0.04		0.02		0.1%	

[1] Kovats retention index (RI). [2] CAS registry number or NIST library number. [3] Percentage of all evening samples in which the compound was detected, with entries > 50% in bold. [4] The mean of emission rates scaled to 100%. [5] Fragment ions relative to *m/z* 91 (100%): 65 (20%), 119 (19%), 162 (11%), 92 (9%), 63 (8%), 89 (6%), 51 (5%). NIST MS Search 'Substructure Information' analysis indicates molecular mass of 162, probable disubstituted phenyl with a carbonyl group.

Table 1.2

Canonical analysis of principal coordinates (CAP) of the effects of species (*Schiedea kaalae* or *S. hookeri*) and time of day on floral scent composition.

(a) ANOVA-like permutation test (*n* = 99999 iterations) of each term.

	df	**SS**	**F**	**P**
Species	1	6.74	52.7	0.00001
Time	1	0.61	4.8	0.00146
Species : Time	1	0.37	2.9	0.02012
Residual	72	9.20		

(b) Compound scores on the first CAP axis, which discriminated between the species. Absolute scores ≥ 0.02 are included. Negative values indicate compounds associated with *S. hookeri*, and positive values indicate compounds associated with *S. kaalae*.

Name	**CAP1 Score**
	S. hookeri
unknown benzenoid	-0.09
indole	-0.08
(E)-hex-2-enal	-0.07
(E)-hex-3-enal	-0.06
methyl 2-aminobenzoate	-0.06
heptane-2,3-dione	-0.06
1,3-dihydro-2-benzofuran	-0.06
benzaldehyde	-0.05
(3E)-4,8-dimethylnona-1,3,7-triene	-0.05
3,5,5-trimethylhex-2-ene	-0.04
anisole	-0.02
N-phenylformamide	-0.02
furan-3-ylmethanol	-0.02
2,2,6-trimethylcyclohexane-1,4-dione	0.06
oct-1-en-3-ol	0.06
4-oxoisophorone	0.09
α-phellandrene	0.12
2-phenylacetaldehyde	0.12
linalool oxide (furanoid)	0.43
linalool oxide (pyranoid)	0.59
linalool oxide (pyranoid) ketone	0.62
	S. kaalae

Supplementary Table 1.S1

Localities of *Schiedea* populations in this study (Wagner *et al.,* 2005). Collections of *S. hookeri* at Waiʻanae Kai were treated as a single population (WK) for this study. Figures are labeled with the population number only.

Species	**Range**	**Location**	**Population collection number**
S. hookeri	Waiʻanae	Kaluaʻa Gulch, S of Puʻuhapapa	Weller and Sakai 879 (BISH, US)
		Waiʻanae Kai, various locations. Ridge separating Waiʻanae Kai and Makaha Valley (Weller and Sakai 794, BISH), ridge separating Makaha and Waiʻanae Valleys (Weller and Sakai 866, US), below Kaʻala (Weller and Sakai 891, BISH, PTBG, US), gulch between the Makaha-Waiʻanae ridge and Puʻukalena (Weller and Sakai 899, US)	WK
S. kaalae	Waiʻanae	Kaluaʻa Gulch, S of Puʻuhapapa	Weller and Sakai 892 (US)
		Pahole Gulch	Weller and Sakai 904 (BISH, PTBG, US)
		E of Puʻukaua, near Puʻumaialau	Takeuchi 3587 (BISH)
	Koʻolau Range	Makaua Valley (Hidden Valley)	Weller and Sakai 881 (BISH, PTBG, US)

Reference

Wagner, W. L., Weller, S. G., and Sakai, A. (2005). Monograph of *Schiedea* (Caryophyllaceae subfam. Alsinoideae). *Systematic Botany Monographs* 72, 1–169.

Supplementary Table 1.S2

Evidence of EAD (electroantennographic detection) responses or attraction of moths for compounds detected by GC-MS. Available online at https://doi.org/10.3389/fpls.2020.01116

CHAPTER 2: Floral scent of artificial hybrids between two *Schiedea* species that share a moth pollinator

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Abstract

Aims

In flowering plants, the ability of pollinators to recognize hybrids through chemical communication may mediate gene flow. Hybrids may produce novel or reduced floral scent blends that are unattractive, or scents similar to one or both parents that remain attractive and promote backcrossing. We characterized the floral scent of artificial F_1 hybrids between two sympatric species pollinated by the same moth species. The importance of hybrid scent for reproductive isolation depends on whether the scent mediates pollinator attraction, so we tested in the field whether moths are attracted by the scent of one species in the absence of visual cues.

Methods

Floral volatiles of artificial F_1 hybrids between Hawaiian *Schiedea kaalae* and *S. hookeri* (Caryophyllaceae) were characterized by dynamic headspace sampling and GC-MS. Behavioral choice tests measured attraction of the moth *Pseudoschrankia brevipalpis* (Erebidae) to *S. kaalae* scent added to inflorescences of relatively unattractive wind-pollinated relatives (*S. kealiae* and *S. globosa*) from the same island.

Results

Most hybrids produced a combination of the distinct sets of floral volatiles from each parent, at intermediate rates of emission. Floral scent did not depend on cross direction, and no novel compounds were detected in hybrids. In the evening, *Pseudoschrankia brevipalpis* preferred inflorescences of wind-pollinated *S. globosa* and *S. kealiae* that were augmented with the scent of 2-3 hidden *S. kaalae* flowers over those that were not augmented.

Conclusions

The intermediate floral scent blends of hybrids, that are produced at similar total emission rates as the parent species, have the potential to attract the moth that is attracted to both parent species, depending on the moth's preferences for precise ratios of compounds. Moth attraction to the floral scent of *S. kaalae* flowers indicates that moths sense and can discriminate the floral scent of this species against a background of volatiles and visual cues from wind-pollinated relatives, showing the importance of scent variation in this genus.

Introduction

A number of reproductive isolating mechanisms maintain the genetic boundaries between plant species, and some barriers act after hybrids are formed by affecting their ability to reproduce. In contrast to the general pattern of flowering plant groups with strong prezygotic barriers (such as distinct phenology and pollinator specificity, Rieseberg and Willis, 2007), later-acting postzygotic barriers can be important for species radiations in cases where closely-related species lack prezygotic barriers to gene flow (Wendt et al., 2008, Widmer et al., 2009). The ability of hybrids to reproduce with other hybrids or parent species could break down species barriers, producing further hybrid generations and backcrosses and slowing or reversing genetic differentiation among taxa. In plants, pollinator attraction is a key component of reproduction accomplished by floral advertisements of rewards, including production of species-specific floral scent blends that are especially useful for attracting nocturnal pollinators (Miyake et al., 1998). The floral scent produced by hybrids in comparison to their parent species could affect attraction of insect pollinators to hybrids, especially if the parental scents are distinct from each other. On one extreme, novel hybrid scents that are unattractive to the pollinators of the parental species but attractive to new pollinators can promote immediate ethological reproductive isolation with

parental species, as shown in natural hybrids of *Orphys* (sexually deceptive orchids, Vereecken et al., 2010) and *Narcissus* (food-rewarding daffodils, Marques et al., 2016). Alternatively, hybrid scent that attracts parental pollinators could enhance backcrossing with one or both parent species, leading to introgression, as shown in a *Yucca* contact zone (Svensson et al., 2016).

The chemical composition of hybrid scent blends could depend on many factors, including the environment (Majetic et al., 2009), the heritability of volatile production (Zu et al., 2015), and the pattern of allelic dominance (Byers et al., 2014). Hybrid individuals studied to date produce volatile blends similar to one parent (Svensson et al., 2016, Zito et al. 2018), quantitatively intermediate blends (Campbell et al., 2016, Svensson et al., 2016), transgressive blends outside the phenotypic range of both parents (Bischoff et al., 2014; Marques et al., 2016, Rubini Pisano et al. 2018), novel volatile compounds (Vereecken et al., 2010, Marques et al., 2016), or reduced total scent emissions (Hirota et al., 2012). The attractive abilities of these scent blends relative to parental species depend on pollinator olfaction and preferences, with some pollinators requiring compounds mixed in precise ratios and others requiring the presence of key compounds (Wright et al., 2005).

In most of these studies, natural hybrid zones have been used to study scent-mediated postzygotic reproductive isolation. These systems can provide insight on local pollinator responses to novel floral phenotypes. However, the natural hybrid population may have already experienced selection for floral traits preferred by local pollinators, leading to a biased sample of hybrids. Natural hybrid populations also include individuals of unknown parentage, including advanced-generation hybrids and backcrosses, so the link between novel genetic combinations and hybrid floral phenotypes is less clear (Bischoff et al., 2014). These problems are partially avoided through study of F_1 hybrids raised from seed in a controlled environment, by eliminating

the selective force of pollinator attraction. Analysis of artificial crosses with known parentage (Hirota et al., 2012; Okuyama, 2015) allows direct assessment of the effects of hybridization on floral volatiles expression, but for food-rewarding pollination systems no studies have directly compared the scent compositions of hybrids of known parentage to those of their parent species.

For the floral scent of hybrids to have an effect on fitness, scent must be used by pollinators for locating or identifying flowers. Insect pollinators are known to integrate olfactory and visual information to find and feed at flowers (Chittka and Raine 2006, Barragán-Fonseca 2020), but in other cases bioassays with synthetic scents have demonstrated that scent alone is sufficient for attraction of some insects (Knudsen et al. 1999, Peakall et al., 2010). In *Manduca sexta* hawkmoths, a suite of visual and olfactory sensory cues operate in tandem at different distances to elicit upwind flight, close-range location of flowers, and feeding behaviors (Raguso and Willis, 2002, Raguso and Willis, 2005, Raguso, 2008, Riffel and Alarcón, 2013). However, little is known about the role of scent versus visual attraction in flowers that lack the conspicuous white petals typical of nocturnal moth pollination, or the nature of sensory modalities used by microlepidoptera to locate flowers. A study of the attraction of microlepidopteran visitors and other diverse taxa to the floral scent of *Silene otites* in the absence of visual cues showed that the floral scent alone attracted a subset of the moth taxa that visited open flowers, which are small and greenish-white (Dötterl et al., 2012). A mix of 11 volatiles of the thistle *Cirsium arvense*, a pollination generalist visited by microlepidoptera as well as bees, wasps, and beetles, was more attractive to moths (increased number of visitors) than the main component (phenylacetaldehyde) alone, but the diversity of moth taxa attracted to the mix and main component were similar (El-Sayed et al. 2008). The endemic lepidoptera of Hawai'i are particularly diverse (Zimmerman, 1958, Ziegler, 2002) and known or inferred to be critical for

pollinating endangered plant populations (Norman et al., 1997, Newbery et al., 1998, Elmore, 2008, Weisenberger et al., 2014, Medeiros, 2015, Shay and Drake, 2018, Aslan et al., 2019, Walsh et al., 2019), but the mechanism of their attraction to flowers has not been studied.

To investigate the potential for hybrid scent to serve as a reproductive barrier via reduced pollinator attraction, we evaluated the floral scent produced by artificial hybrids of two sympatric apetalous Hawaiian plant species, *Schiedea kaalae* and *S. hookeri* (Caryophyllaceae). These species share an endemic microlepidopteran pollinator but have no record of producing hybrids where their ranges overlap (Wagner et al., 2005, Willyard et al., 2011) and produce qualitatively distinct floral scents in the evening (Weisenberger et al., 2014, Weller et al. 2017, Powers et al., 2020). We determined whether the scent blends of artificial F_1 hybrids are identical to one parent, outside the range of either parent, intermediate, reduced, or novel. Using field experiments, we established whether the evening floral scent of *S. kaalae* in the absence of visual cues is sufficient to attract the pollinator of both species against a background of floral scent from either of two relatively unattractive wind-pollinated relatives. Understanding the potential for species-specific olfactory cues in attracting pollinators is necessary to interpret the evolution of floral scent in this island-endemic genus. Even in the absence of species-specific olfactory cues, if volatiles emitted in the evening are important for moth attraction, reduced or altered floral scent production in hybrids may lower hybrid fecundity and contribute to maintaining the species boundary.

Materials and Methods

Hybridization in Schiedea

Schiedea is a monophyletic genus that has radiated across the Hawaiian Islands and includes 32 extant species with diverse pollen vectors (moths, wind, and presumably birds) and breeding

systems (e.g., hermaphroditism, dioecy, autogamy; Wagner, et al., 2005). Early-acting postzygotic viability barriers are generally weak in the genus: all tested crosses between 17 species produced seeds and vigorous F_1 progeny (Weller et al., 2001). Some *Schiedea* species may produce hybrid zones: out of ten instances of current sympatry (Wagner et al., 2005), five instances of extant natural hybridization have been observed based on morphology (Weller et al., 2001), and seven instances of current and historical introgression between *Schiedea* species have been detected through molecular methods (two of the seven were already known from morphological intermediates, Soltis et al., 1996, Wallace et al., 2011; Willyard et al., 2011).

Focal species

Schiedea kaalae Wawra (sect. *Mononeura*) and *S. hookeri* A. Gray (sect. *Schiedea*) are hermaphroditic, self-compatible, protandrous, perennial herbs or subshrubs (respectively) native to Oʻahu, Hawaiʻi, USA, where populations of the two species occur in sympatry in parts of the Waiʻanae Mountains (*S. kaalae* [410 - 730 m above sea level, asl] and *S. hookeri* [260 - 870 m asl], Wagner et al*.,* 2005) and can flower at the same time. *Schiedea kaalae* also occurs in the Koʻolau Mountains (Wagner et al*.,* 2005). These species occur in different clades that diverged c. 1.3 Mya based on a root age of 7.3 Mya for the genus (Willyard et al*.,* 2011). Both species are listed as endangered by the US Fish and Wildlife Service and critically endangered by the IUCN (Ellshoff et al*.,* 1991; Bruegmann and Caraway, 2003; Wagner et al*.,* 2005 *a,* Bruegmann et al., 2016), and a total of only about 28 *S. kaalae* individuals in five populations remained in the wild before restoration efforts (Weisenberger et al*.,* 2014). *Schiedea hookeri* is more common in nature than *S. kaalae*, and large populations also exist following restoration efforts (D. Sailer, personal communication). Both species possess similar floral morphology with reflexed sepals 3-4 mm long, no petals, 10 stamens, 3 styles, and 5 nectaries (Wagner et al*.,* 2005).

When artificially crossed, F_1 hybrid seeds between sympatric *Schiedea kaalae* and *S. hookeri* germinate and flower in greenhouse conditions. However, despite overlap in geographic range and phenology, and a shared pollinator species (Wagner et al., 2005, Weller et al., 2017, see below), no evidence of natural hybridization or past genetic introgression between these taxa has been detected (analysis of eight plastid and three nuclear loci; Willyard et al., 2011), suggesting that postzygotic barriers that affect hybrid viability or fecundity may exist. The inability of hybrids to attract pollinators is one hypothesis for the lack of hybrid populations or genetic introgression in areas of sympatry between *Schiedea kaalae* and *S. hookeri*. Other postzygotic barriers may also explain the lack of a hybrid zone in this species pair. For example, if hybrid germination, survival, pollen viability, or seed viability is low, the growth and flowering of first or second generation hybrids would be impaired (Reiseberg and Willis 2007) and prevent hybrid scent from affecting reproductive isolation.

Schiedea pollination and floral scent

Pollination by native microlepidoptera has been observed in two species of *Schiedea* (Weller et al., 2017). Most *Schiedea* species have specialized tubular nectary extensions (Harris et al., 2012) that are probed by moths to remove nectar (Weller et al., 2017). The two focal species of this current study, *Schiedea kaalae* and *S. hookeri*, are pollinated by the moth *Pseudoschrankia brevipalpis* (Erebidae; Weller et al., 2017). *Pseudoschrankia brevipalpis* exhibits flight patterns characteristic of moths searching for floral scent. In addition, when beginning their foraging activity in the evening, moths fly upwind as they approach the experimental populations and can navigate to inflorescences that are out of sight (A. Sakai, personal observations). Despite sharing a pollinator, *S. kaalae* and *S. hookeri* produce distinct scent blends from each other, dominated by different compound classes, but also share some volatiles in common (Powers et

al., 2020). Many volatiles produced by both species show a peak evening output that corresponds to the time of peak pollinator activity, and include common insect attractants such as indole, phenylacetaldehyde, and linalool derivatives (Powers et al., 2020). At a site planted exclusively with *S. kaalae*, *P. brevipalpis* preferred *S. kaalae* inflorescences (69% of inflorescence visits in choice tests, Weller et al., 2017) over *S. hookeri* inflorescences, demonstrating that moths can discriminate between the species.

In addition to discriminating between *S. kaalae* and *S. hookeri*, *P. brevipalpis* prefers to visit either of those species that develop relatively large nectaries over two related wind-pollinated species that occur on the same island (O‘ahu; *S. kealiae* and *S. globosa*, Weller et al., 2017). The two wind-pollinated species have small vestigial nectaries and produce scents that are distinct from the scents of the two moth-pollinated species (Jürgens et al., 2012).

Floral scent of hybrids

Crossing design and volatiles sampling

Volatile emissions were measured in the evening on plants of *Schiedea kaalae*, *S. hookeri*, and F_1 hybrids using the protocols for greenhouse cultivation, dynamic headspace collection, GC-MS, and data processing methods used in Powers et al. (2020) and summarized here. We used the same evening samples collected for the two parental species in this paper. Some plants of the two species were grown from seeds or cuttings of six populations from the Wai‘anae Mountains (10 *S. kaalae* and 10 *S. hookeri* plants, Appendix 2.S1; all collections were made before species were listed as federally endangered in 1991 and 1996, for *S. kaalae* and *S. hookeri,* respectively). Plants also were grown from intraspecific and interspecific (hybrid) crosses between cultivated plants from these populations (see Table 2.1 for sample sizes). To

produce the plants of *S. kaalae* and *S. hookeri*, we used intrapopulation and interpopulation crosses within species because most natural populations now consist of a single individual and are highly inbred (Weisenberger et al., 2014). We conducted crosses between populations in both directions (e.g. with a plant from one species acting as the maternal parent and the plant from the other species acting as the paternal parent and vice versa) to examine potential nuclear-cytoplasmic interactions or the direct effects of the two species' cytoplasmic genomes on floral scent (hereafter, cytoplasmic effects), which to our knowledge have not been studied before. In the absence of these interactions for scent production, scent composition and total emission rates will be similar for both cross directions. The hybrid crosses are denoted with the initial of the maternal parent first, e.g., H x K for a hybrid with *S. hookeri* as the maternal parent and *S. kaalae* as the paternal parent. The species (interpopulation crosses) and hybrids produce the following numbers of flowers per inflorescence in the greenhouse (median and interquartile range, n = 859 plants): *S. kaalae* 332 (184 - 504), *S. hookeri* 26 (19 - 34), K x H hybrids 126 (67 - 237), H x K hybrids 201 (98 - 336).

Scent samples were collected from November 2016 - April 2017 in the greenhouse to provide a common environment for observing genetic differences. Conditions in the field may differ in light level, temperature, and humidity, which could affect the expression of scent phenotypes. Because scent composition and amount depend strongly on time of day in both species (Powers et al., 2020), only samples taken 0 - 4.5 hr after sunset (mean ± standard deviation 2.0 ± 0.9 hr after sunset, corresponding to 17:15 - 21:45 local time) were used. This corresponds to the timing of moth activity in both species (Powers et al. 2020). Dynamic headspace samples of floral volatiles were taken by enclosing inflorescences in oven bags, allowing volatiles to equilibrate for 30 min at 20 - 26 °C and pumping for 30 min through a Tenax scent trap with a pre-trap flow rate of 200 mL/min. The numbers of open male- and female-phase flowers, closed

(post-anthesis) flowers, and floral buds were recorded immediately after sampling. All plants chosen had ≥ 10 open flowers. To assess the magnitude of intraplant variation and variation between dates, for 13 plants multiple samples were taken from different inflorescences on different dates. Because these samples yielded similar scent emissions (data not shown), they were averaged within a plant before statistical analysis. Ambient controls (n = 19) were taken from an empty oven bag sampled for the same duration to identify contaminants (see below). Floral scent emissions were quantified by thermal desorption gas chromatography-mass spectrometry (TD-GC-MS; see Powers et al., 2020 for details). Volatile emission rates were calculated within each compound class from peak integrations by calibration with dilutions of authentic standards. We analyzed the same filtered set of compounds as in Powers et al. (2020). Emission rates were standardized by the number of open flowers.

Statistical analysis of floral scent

To determine how scent intensity in hybrids related to the parent species, the total emission rates per flower of the two species and two cross directions were compared. A difference between the cross directions would indicate cytoplasmic effects (see methods). If genetic control of floral volatiles emissions is additive (no effects of dominance or epistasis), then we predict total emission rates in hybrids will be equal to the mean of the emission rates of the two parental species. We tested this null hypothesis using the *multcomp* R package (Hothorn et al., 2008, R Core Team, 2018). To analyze patterns of scent composition among plants, we performed a non-metric multidimensional scaling (NMDS) with Bray-Curtis distances between relative volatile emission rates (after applying a square-root transformation) using the *vegan* R package (Oksanen et al., 2018). To see whether levels of beta diversity (variation in relative scent compositions among plants) differed among the two parent species and hybrids, we

conducted an analysis of multivariate homogeneity of group dispersions using the *betadisper* function of *vegan* with Bray-Curtis distances. To test whether the hybrid cross directions differed in relative scent composition, a PERMANOVA was conducted between the two cross directions using the *adonis* function of *vegan*. To compare emission rates of individual volatiles between the groups in more detail and identify which volatiles were correlated with each other we visualized the log emission rate per flower of each volatile in a heatmap, and grouped volatiles by WPGMA hierarchical clustering analysis of Pearson distances. The clustering analysis identifies sets of volatiles that have similar patterns among and within experimental groups, which could indicate shared biochemical regulation or genetic correlations. The heatmap is useful for assessing which volatiles are present in each group, as well as their emission rates and rarity.

Behavioral tests of moth preference

One way of testing whether a pollinator uses scent is to remove visual cues like color, size and shape by enclosing flowers in bags or other structures to hide them. We chose this general approach, enclosing flowers in mesh bags, similar to the approach of several previous studies in other systems (Knudsen et al., 1999, Raguso and Willis, 2002, Raguso and Willis, 2005, Dötterl et al., 2012, Riffel and Alarcón, 2013, Barragán-Fonseca 2020). Such an approach isolates the role of scent, although it does not determine exactly which compounds are attractive, as can be done by enclosing synthetic scent blends emitters within mesh bags (e.g., El Sayed et al., 2008). Such an experiment could be the next step, to identify behaviorally active compounds in each species.

We used choice tests to assess whether the evening scent of *S. kaalae* attracts moths against a background of the scent and visual cues of one of two wind-pollinated species (*S. kealiae* or *S.*

globosa) whose inflorescences were previously shown to be relatively unattractive to moths in choice tests (Weller et al., 2017). Choice tests experiments were performed at a c. 20 x 20 m experimental outplanting of *Schiedea kaalae* plants spaced c. 0.5 m from each other in ʻĒkahanui Gulch, Oʻahu, Hawaii, USA (described in Weisenberger et al., 2014 and Weller et al., 2017). Visitation to choice tests likely reflects short-distance (< 10 m) attraction of moths already foraging in the patch, where they might already be attracted from the larger plume of *S. kaalae* floral odor. Choice sticks were constructed as in Weller et al. (2017). The apparatus consisted of two florist tubes containing *S. globosa* or *S. kealiae* inflorescences of the same species and sex and approximately the same size taped to the ends of a 0.45 m horizontal stick, with a perpendicular 1 m stick to serve as a handle. Inflorescences of both sexes of *Schiedea kealiae* (Weller & Sakai 791, Kealia Trail, cultivated) and *S. globosa* (Weller & Sakai 844, Makapu'u, natural) were provided by Scott Heintzman and Alex Loomis, respectively. We hid *S. kaalae* flowers in mesh bags that allowed the passage of volatiles. A portion of a *S. kaalae* inflorescence with 2 or 3 flowers (cut from plants at the experimental outplanting site) was inserted into the florist tube on one side of the choice stick and then enclosed in a white mesh organza bag (mesh size 0.37 mm, 27 holes per cm) to shield it from view. A similar empty bag was placed on the opposite side as a control in case the white bag was attractive. For each side of the choice stick, we recorded moth approaches (flight within 10 cm), visits (approaches and landings on the exposed inflorescence of *S. globosa* or *S. kealiae* or on the mesh bag). We performed 10 choice tests (16 observer h) with *S globosa* and 18 choice tests (25 observer h) with *S. kealiae* over 6 nights from March 30 to April 4, 2019. Each choice test was observed for at least 30 min between 17:30 and 20:00 HAST, after the first moth was sighted within the outplanting site. To assess preference for *S. kaalae* scent, the proportions of approaches or visits were estimated with generalized linear models using the R package glmmTMB (Brooks et

al., 2017). We assumed a beta-binomial distribution to account for overdispersion, and used each choice test as the unit of replication, weighting by the total number of visits to that choice test. A generalized linear hypothesis test tested the null hypothesis that the mean is 50% using the R package *emmeans* (Lenth et al. 2018).

Results

Floral scent of hybrids

The total volatiles emission rate per flower of hybrids was not significantly different from the emission rates of *Schiedea kaalae* or *S. hookeri* based on Tukey tests (Figure 2.1). The total emission rates of both cross directions was not significantly different from the mean of the total emission rates of the two species ($p = 0.55$ for K x H and $p = 0.97$ for H x K). In hybrids the cross direction did not have an effect on total emission rate (Tukey post-hoc test, $p = 0.89$). The number of compounds emitted did not differ among parents and hybrids (Figure 2.1), but the Shannon diversity index (which captures richness as well as evenness), was higher in *S. hookeri* compared to hybrids and *S. kaalae* (Figure 2.1). The parent species produced qualitatively distinct scent blends (Figure 2.2, Figure 2.3, Powers et al., 2020), with the scent of *S. kaalae* dominated by three cyclic linalool oxides (67% of the total blend) with relatively minor amounts of aliphatics and phenylacetaldehyde, and the scent of S. hookeri composed of oct-1-en-3-ol (41%) with a diverse set of aliphatics, benzenoids, and indole as minor constituents. As a group, F_1 hybrid plants produced a set of volatiles drawn from the typical volatiles of both parent species (Figure 2.2), and no novel volatiles were produced. In particular, a majority of hybrids produced the volatiles benzaldehyde, hexanal, octan-3-one, and oct-1-en-3-ol produced by both species, the three linalool oxides and 4-oxoisophorone produced primarily by *S. kaalae*, and indole, an unknown benzenoid, and 2,3-heptanedione produced

primarily by *S. hookeri* (Figure 2.2, Appendix 2.S2). For a given compound, the absolute emission rates in hybrids were generally intermediate to the emission rates of the two parents (Figure 2.2, Appendix 2.S2). However, the volatiles that were emitted by both species in similar amounts (the "mushroom" aliphatics 1-octen-3-ol and 3-octanone) were emitted at higher median amounts in hybrids than either parent species (Appendix 2.S2). Other volatiles were produced by one parent but rarely occurred in hybrids or were present in low amounts: the carotenoid derivative 2,2,6-trimethylcyclohexane-1,4-dione was common in *S. kaalae* and the homoterpene (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was common in *S. hookeri* but each was rare in hybrids (Figure 2.2).

Most hybrids produced relative scent compositions intermediate to those of the two species, although a few hybrids produced scent compositions that overlap with the range of parents scents, based on the NMDS analysis (Figure 2.3). The hybrid plants showed a magnitude of interplant variation (average Bray-Curtis distance to the centroid 0.24) similar to *S. kaalae* (0.20), and lower than the interplant diversity of *S. hookeri* (0.40, analysis of multivariate homogeneity of group dispersions $p < 0.001$, Figure 2.3). There was no general separation of scent composition by hybrid cross direction (which species served as the maternal parent, PERMANOVA $p = 0.54$, Figure 2.3). Resamples of individuals (averaged in other analyses) yielded similar scent compositions (Appendix 2.S3).

Some volatiles were highly correlated with each other across plants (Figure 2.2), which could indicate they are under shared regulation, are genetically correlated, or merely co-occur in one species or the other. For example, all the monoterpenes (including the linalool oxides) and 2-phenylacetaldehyde cluster together (Figure 2.2, first block from the top), which could be due to common regulation or the fact that they occur together in *S. kaalae* and hybrids but are

largely absent from the scent of *S. hookeri*. Most of the volatiles in the fourth block of volatiles occur in *S. hookeri* and hybrids but not *S. kaalae*, and these compounds originate from diverse biochemical pathways (green-leaf aliphatics, benzenoids, and nitrogenous compounds, Dudareva et al., 2004).

Moth attraction to floral scent

We tested the attractive ability of the scent of *Schiedea kaalae* against empty bag controls, in a total of 28 choice tests that spanned 40 observer-hr. Moths preferred to approach (77%) and visit (73%) *S. globosa* inflorescences augmented with *S. kaalae* scent (percentages of interactions with the *S. kaalae* side of the choice stick), and approach (59%) and visit (74%) *S. kealiae* augmented with *S. kaalae* scent compared to unaugmented controls (Figure 2.4). This preference was statistically significant at $\alpha = 0.05$ for approaches but not visits (Figure 2.4). Moths only approached and visited 57 times compared to approaching without visiting 228 times. Depending on the wind-pollinated species used and whether the inflorescence was augmented with *S. kaalae* scent, only 14 - 23% of approaches led to visits.

Discussion

Moths attracted to floral scent

We established experimentally that the scent of *Schiedea kaalae* is attractive to the endemic Hawaiian moth *Pseudoschrankia brevipalpis,* as has been demonstrated for other microlepdopteran pollinators using floral scent or artificial scent mixtures with visual cues blocked (Dötterl et al., 2012, El Sayed et al. 2008). Because the flowers were bagged, specific visual signals (the appearance of *S. kaalae* inflorescences or flowers) were probably not required for approaches or visitation in this moth species. However, stronger tests of this

hypothesis would require application of artificial or extracted scents from *S. kaalae* because the mesh we used may not have completely hidden the flowers from the moths. In addition, a small number of *S. kaalae* flowers were able to attract moths against a background of competing scent and visual presentation from wind-pollinated inflorescences that have been previously shown to be relatively unattractive to these moths (Weller et al., 2017). These results indicate that moths can use the species-specific scent of *S. kaalae* to locate flowers in this food-rewarding pollination system. Most volatiles that *S. kaalae* produces in the evening are typical of moth-pollinated taxa in other plant families (Powers et al. 2020), so it is likely that they play a role in long or short-distance attraction.

In our experiment, discrimination by moths between wind-pollinated inflorescences augmented with *S. kaalae* and controls without the *S. kaalae* flowers was not complete, with moths sometimes approaching (23 - 41% of the time) or visiting (26 - 27%) the unaugmented infloresences. Probably due to the low sample size, this preference was not statistically significant for visits. The incomplete preference could be due to the diffuse *S. kaalae* scent present in the population, or any vestigial attractive ability of the wind-pollinated species inflorescences (visual or olfactory). During transitions to wind pollination within genera, wind-pollinated taxa are known to lose volatiles that provoke antennal responses in pollinators (Wang et al., 2019).

Moths visited (landed on the bag or wind-pollinated inflorescence) only 14 - 23% of the time that they approached a side of the choice stick, indicating that their landing behavior may be suppressed by the unfamiliar or unattractive visual cues of the wind-pollinated inflorescences, or the discordance between these visual cues and the scent cues of *S. kaalae*. A measurement of the proportion of approaches that result in visits to unbagged *S. kaalae* flowers is necessary to

confirm this hypothesis. Further studies with bagged flowers could confirm that feeding behaviors (and thus pollen export or import) can also be induced by scent alone. In rare cases, we observed the head-bobbing motions normally associated with feeding on *Schiedea* nectaries (Weller et al., 2017) when moths landed on the mesh bags with *S. kaalae* flowers inside them, but this was not systematically documented in this study. In *Manduca sexta* hawkmoths, a combination of visual and scent cues are required to achieve feeding behaviors (Raguso and Willis 2002, Raguso and Willis 2005).

Experiments that test the attractive ability of single volatiles or sets of volatiles could identify the components of the scent blend necessary for moth attraction, and if successful provide a rapid bioassay for assessing pollinators at potential restoration sites. These tests would also reveal whether moths are attracted to volatiles shared across moth-pollinated *Schiedea*, or if they use species-specific compounds. A study of *Cirsium* volatile mixes showed that while one compound (phenylacetaldehyde, which increases in *S. kaalae* in the evening), was sufficient to attract microlepidoptera, the addition of minor compounds increased visitation (El-Sayed et al. 2008). It follows that both single general attractants and other reinforcing volatiles may be important for moth attraction. Finally, the preference for *S. kaalae* over *S. hookeri* at a site planted with *S. kaalae* suggests the hypothesis that scent or visual cues are learned, i.e., preferences are produced or reinforced by associating specific floral traits with a reward. Hawkmoths learn to associate nectar more strongly with olfactory cues than visual ones, although the presence of both reduces decision times (Riffel and Alarcón, 2013).

Hybrid scent is intermediate between parents

As a group, hybrids of sympatric *Schiedea kaalae* and *S. hookeri* produce nearly all of the compounds that are shared by or unique to each parental species, at similar total levels and

numbers of compounds per plant, without producing novel volatiles. Individual hybrid plants vary in which volatiles they produce, but are mostly intermediate in scent composition, with a few individuals resembling either parent species. These results suggest that simultaneous production of the volatiles of both species is biochemically and genetically possible and that synthetic or regulatory genes from each parent are active in hybrids. Neither total scent emissions nor scent composition depended on the direction of the cross, indicating that cytoplasmic effects were absent, and so hybrids germinating in populations of either species would have similar floral scents. Thus far, only nuclear genes have been reported to affect scent production (Borpghi et al., 2017), which is consistent with this result.

Scent reduction or novelty that makes hybrids unrecognizable or attractive to a new species of pollinator have been identified as postzygotic reproductive barriers (Marques et al., 2016), but those two possibilities can be ruled out in hybrids of this *Schiedea* species pair. The implications for reproductive isolation are still unclear because the attractive ability of each individual volatile or suite of volatiles has not yet been established. However, if moths (*Pseudoschrankia brevipalpis*) are able to respond to the volatile blend of each species regardless of competing signals from the other species, then hybrids that produce mixture of the two species' scents should still be attractive to moths. The choice test experiments with bagged *S. kaalae* suggest that moths may have this ability since they were attracted to *S. kaalae* scent when mixed with scent from a wind-pollinated species, which are both distinct from *S. kaalae* (Jürgens et al., 2012, Powers et al., 2020). Intermediate scent blends (and some blends occasionally overlapping with the scent of a parental species) were also observed in natural hybrids of two species of Joshua tree (*Yucca brevifolia* sensu lato) at a secondary contact zone, and genetic evidence of backcrossing indicates that moths must have visited those hybrids (Svensson et al., 2016). In two orchids that converged in behaviorally active scent compounds to attract the same

pollinator by sexual deception, hybrids were also able to attract that pollinator because they produced the same set of active compounds as the parent species, although the blend of non-active compounds was intermediate in hybrids (Cortis et al. 2009). Alternatively, effects of intermediate blends may be unexpected: hybrids of two bee-pollinated sexually deceptive orchids produced a combination of parental volatiles (and two new compounds out of 73), which made them attractive to a new butterfly pollinator (Vereecken et al., 2010). Ultimately, choice tests of *Schiedea* hybrids versus parents are required to confirm that hybrids are not reproductively isolated from their parents by their intermediate scent blend (Ma et al., 2016).

While we studied a case where reproductive barriers have evidently stopped the formation or persistence of hybrids, a comparative study with other *Schiedea* hybrid zones (Weller et al., 2001) and artificial hybrids generated from zones of sympatry that lack hybrids may yield different patterns of parental versus hybrid scent. For example, gynodioecious, wind-pollinated *S. salicaria* has produced a hybrid swarm with hermaphroditic *S. menziesii* which is presumably biotically pollinated but currently selfing (Wallace et al. 2011). Due to asymmetric gene flow, natural hybrids have a nuclear genome, morphology, and sex expression similar to *S. salicaria*, but the chloroplast genome of *S. menziesii* (Wallace et al. 2011). We predict that hybrids would also have a floral scent similar to *S. salicaria* due to replacement of the nuclear genome, and if so, be less attractive to any biotic pollinators. In other cases, particularly on older islands, the high genetic distance between species may cause hybrids to have genetic incompatibilities that result in reduced floral scent and represent a postzygotic reproductive barrier, if the species are biotically pollinated.

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Figures and Tables

Figure 2.1

Total volatile emissions rates per flower, number of compounds, and Shannon diversity index for *Schiedea kaalae*, *S. hookeri*, and reciprocal hybrids (denoted by maternal then paternal parent). Quartiles and medians are indicated by boxplots. Differences in means at the α = 0.05 level are indicated by Tukey letterings above each box.

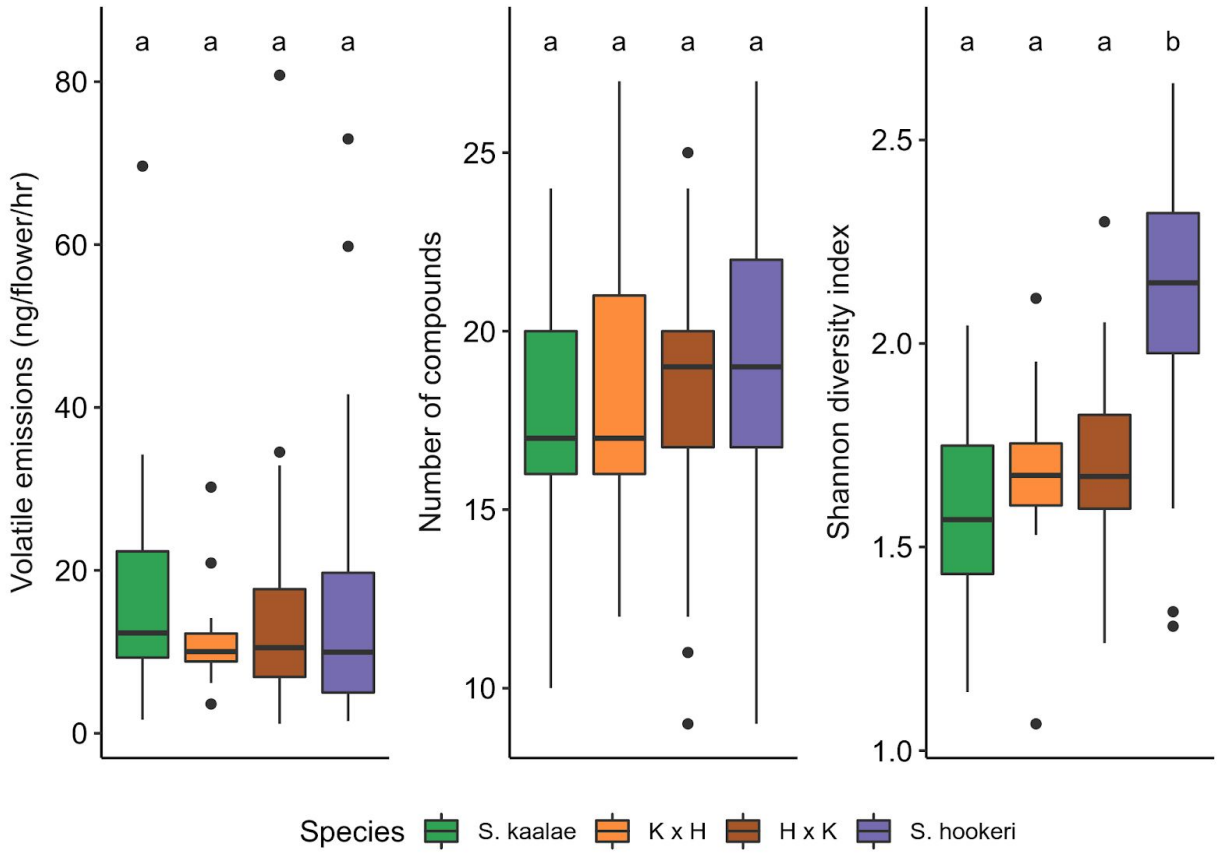


Figure 2.2

Patterns of floral volatiles in *Schiedea kaalae*, *S. hookeri*, and reciprocal hybrids. The emission rate per flower is shown by color (increasing from black to purple to yellow). Columns represent evening samples (1 - 4 samples per plant) and are ordered within the species or hybrid groups by scores along a one-dimensional NMDS ordination. Rows correspond to individual volatile compounds (that occur in >13% of the samples), and volatiles are grouped by WPGMA hierarchical clustering analysis of Pearson distances. The clustering reflects how volatiles are correlated within and across sampling groups.

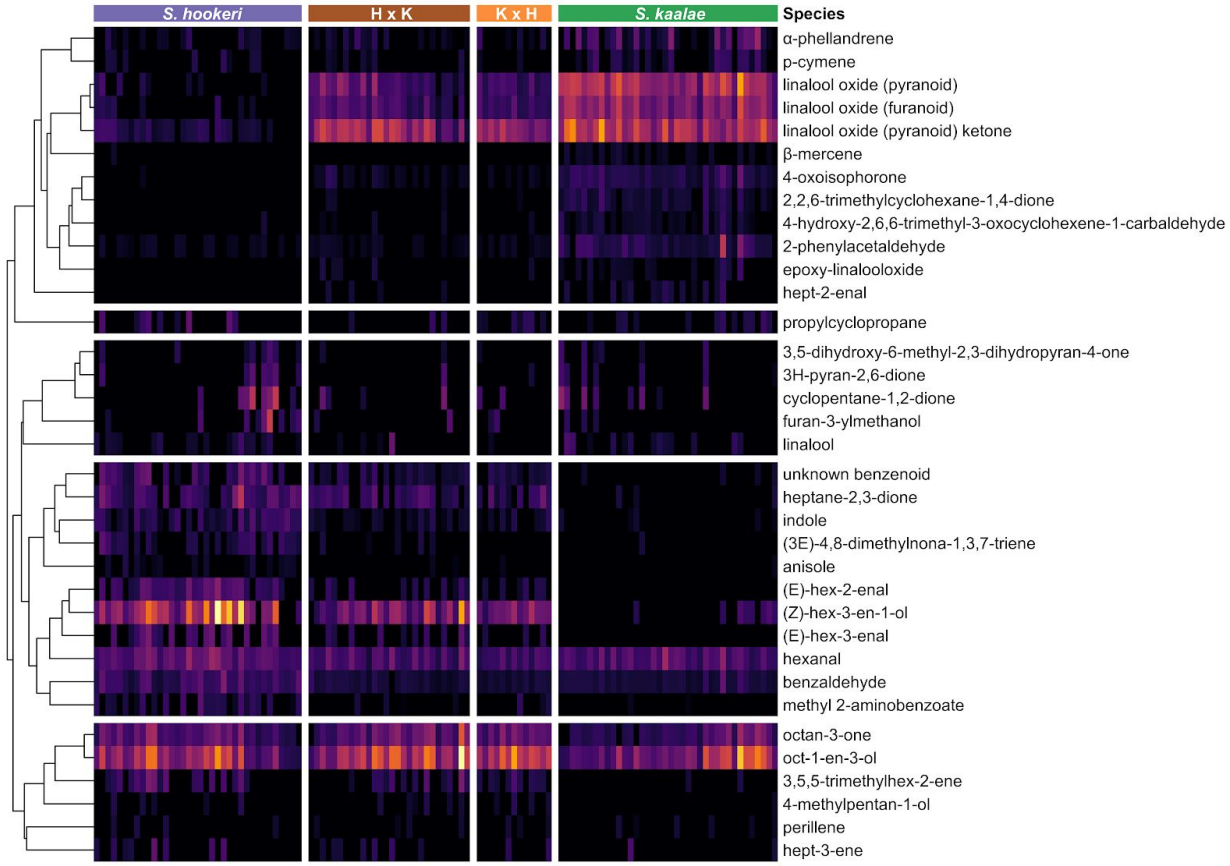


Figure 2.3

Differences in floral scent composition of evening plants of *Schiedea kaalae*, *S. hookeri*, and reciprocal hybrids, visualized by NMDS of Bray-Curtis dissimilarities between square-root transformed relative emission rates (stress = 0.11). Samples are connected by lines if collected from different individuals of the same genotype (produced by propagation), or the same cross, defined by exact parentage. Volatiles that occured in more than 20% of samples are labeled at their weighted position in the ordination.

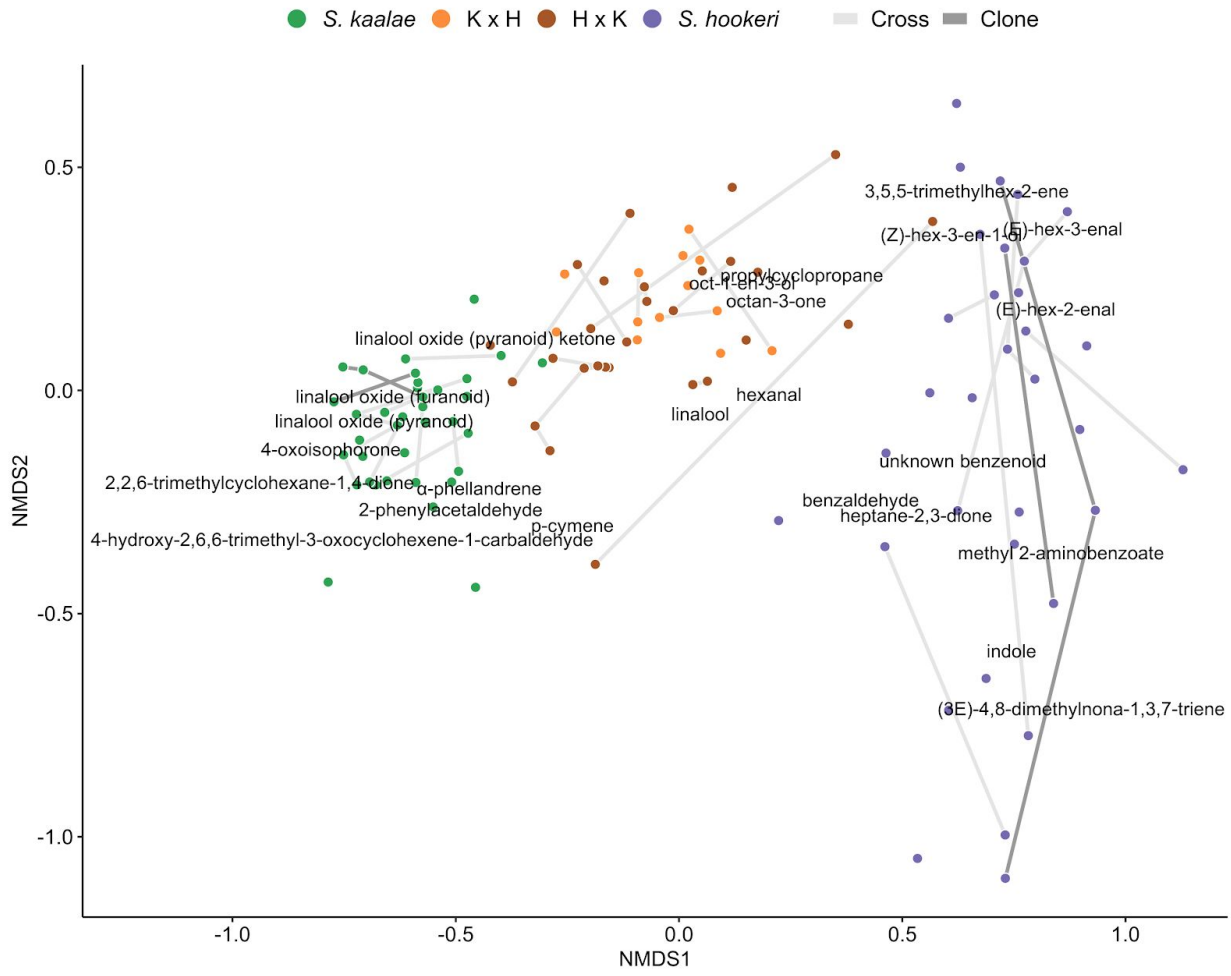


Figure 2.4

Preferences of the moth *Pseudoschrankia brevipalpis* for wind-pollinated *Schiedea* inflorescences (either *S. globosa* or *S. kealiae*) augmented with bagged *S. kaalae* versus controls with empty bags. P-values test the null hypothesis of no preference (μ = 50%, dashed line) according to a beta-binomial generalized linear model. Bars indicate the means and standard error of the proportion of approaches (light grey) or visits (dark grey). The number of interactions on the side with bagged *S. kaalae* is expressed as a fraction of the total at the bottom of the bar. With *S. globosa*, there were 10 choice tests over 16 observer-hr, and for *S. kealiae* there were 18 choice tests over 15 observer-hr.

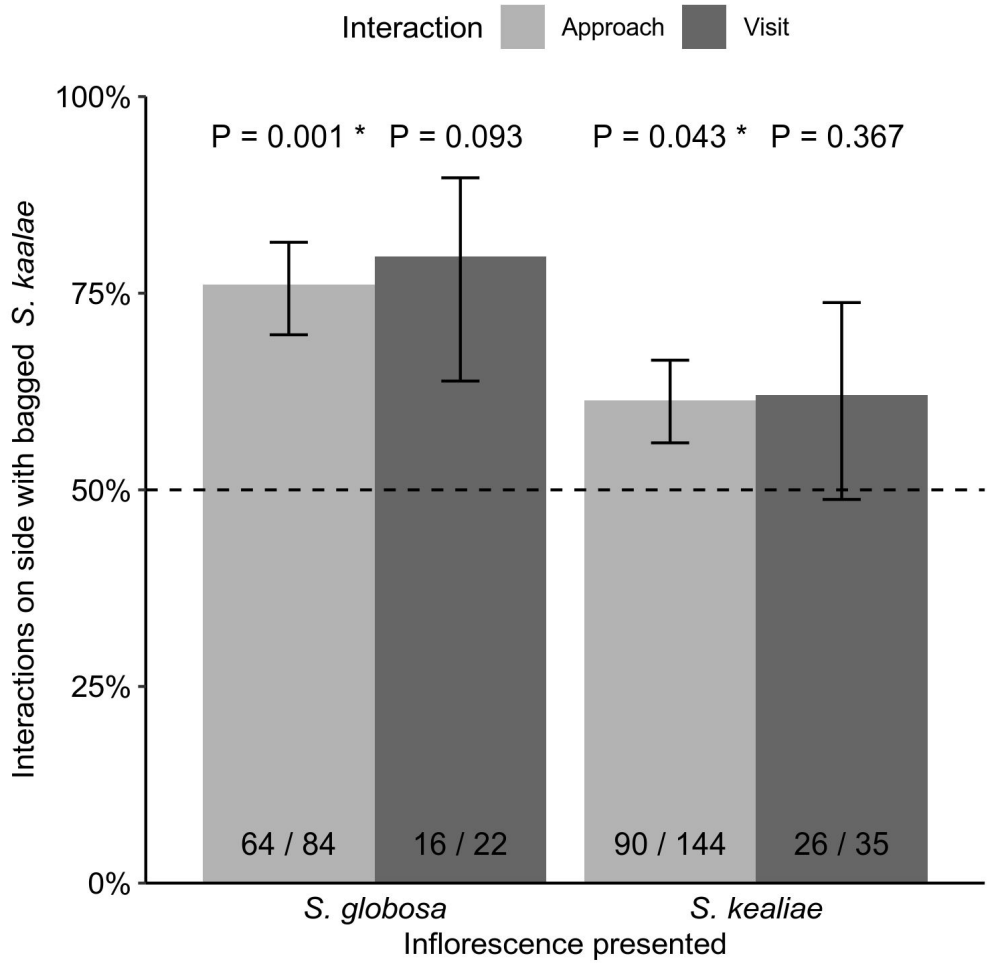


Table 2.1

Schiedea plants sampled for floral volatiles (n = 32 *S. hookeri*, 12 *S. kaalae* x *S. hookeri*, 21 *S. hookeri* x *S. kaalae*, 32 *S. kaalae*), organized by their maternal and paternal parents. The number of maternal (n = 19) or paternal plants (n = 22) used to make the crosses are given in parentheses next to the population number. On the diagonal, the number of plants from within-population crosses is given first and the number of sampled plants that originated from the field (as seeds or cuttings) is given second. Shading indicated the cross type. All populations occur in the Wai‘anae Range except 881 which occurs in the Ko‘olau Range (see Appendix 2.S1 for population localities).

		Paternal					
		S. hookeri		***S. kaalae***			
Maternal		879 (4)	WK (8)	3587 (4)	892 (3)	904 (3)	881 (0)
S. hookeri	879 (4)	10 + 1	2	3	1	3	
	WK (4)	4	6 + 9	4	6	5	
S. kaalae	3587 (5)	2	3	5 + 4		3	
	892 (3)			3	3 + 1	1	
	904 (3)	4	2	2	1	4 + 2	
	881 (0)						3

Appendix 2.S1

Localities of *Schiedea* populations in this study (Wagner *et al.,* 2005). Collections of *S. hookeri* at Wai‘anae Kai were treated as a single population (WK) for this study.

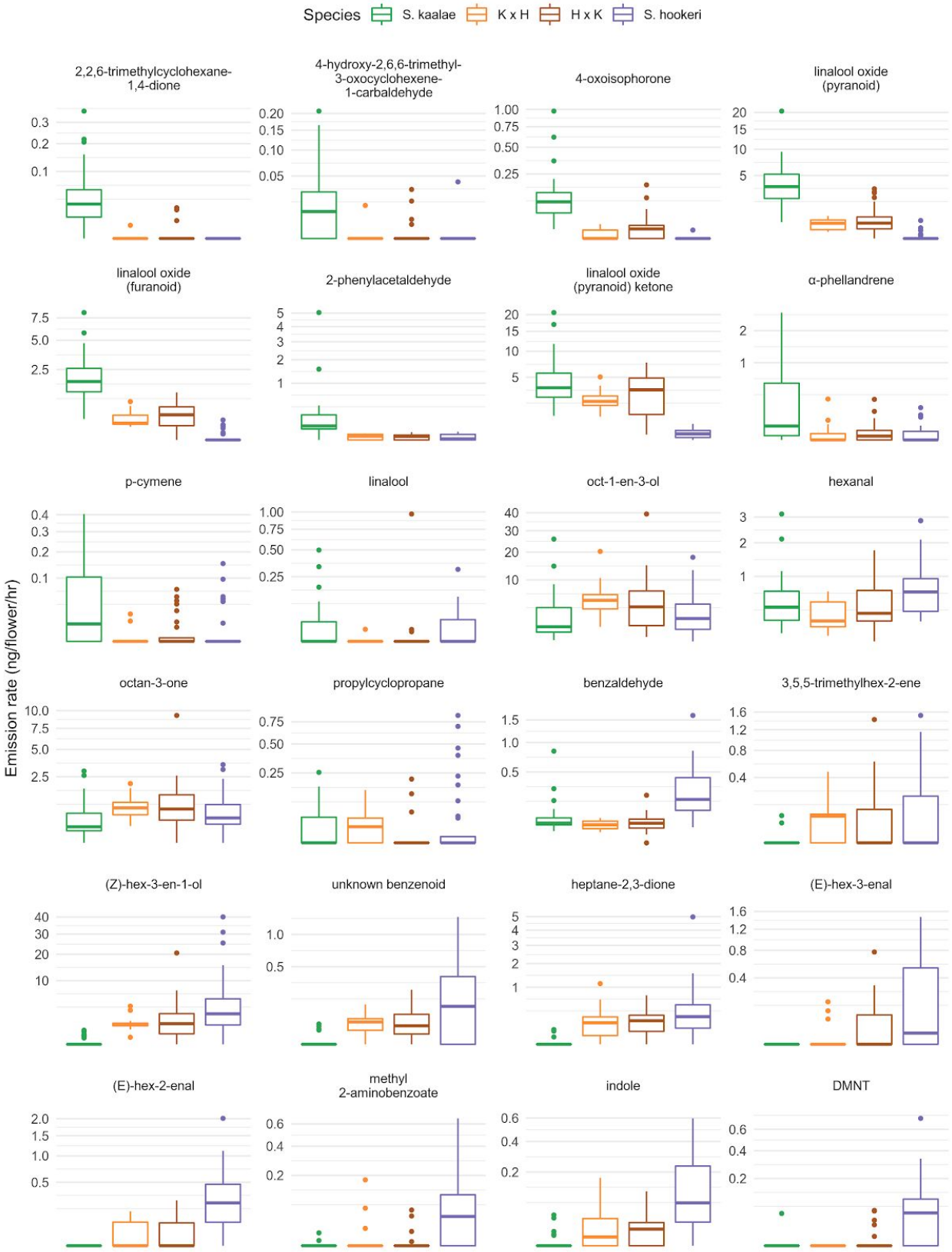
Species	Range	Location	Population collection number
S. hookeri	Wai‘anae	Kalua‘a Gulch, S of Pu‘uhapapa	Weller and Sakai 879 (BISH, US)
		Wai‘anae Kai, various locations	WK (Weller and Sakai 794, BISH; Weller and Sakai 866, US; Weller and Sakai 891, BISH PTBG, US; Weller and Sakai 899, US)
S. kaalae	Wai‘anae	Kalua‘a Gulch, S of Pu‘uhapapa	Weller and Sakai 892 (US)
		Pahole Gulch	Weller and Sakai 904 (BISH, PTBG, US)
		E of Pu‘ukaua, near Pu‘umaialau	Takeuchi 3587 (BISH)
	Ko‘olau Range	Makaua Valley (Hidden Valley)	Weller and Sakai 881 (BISH, PTBG, US)

Reference

Wagner, W. L., Weller, S. G., and Sakai, A. (2005). Monograph of *Schiedea* (Caryophyllaceae subfam. Alsinoideae). *Systematic Botany Monographs* 72, 1–169.

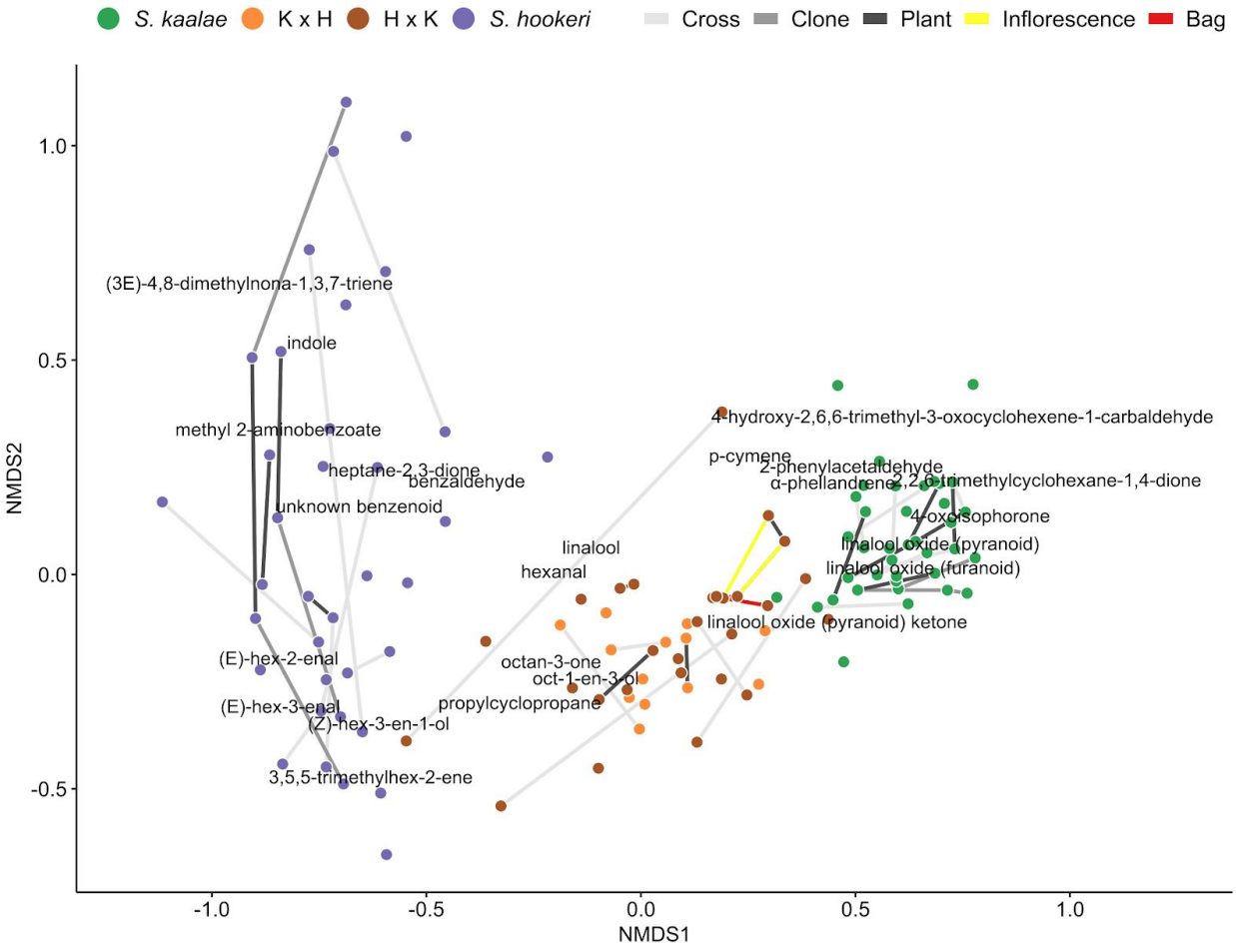
Appendix 2.S2

Volatile emission rates per flower (square root scale) for *Schiedea kaalae*, *S. hookeri*, and reciprocal hybrids (denoted by maternal then paternal parent). Volatiles occurring in > 20% of samples are included. Boxplots show quartiles and medians. Volatiles are arranged (left to right and top to bottom) by their position in a 1-dimensional NMDS ordination to show compounds that are unique to each species at each end.


Species
S. kaalae
K x H
H x K
S. hookeri
Emission rate (ng/flower/hr)
2,2,6-trimethylcyclohexane-1,4-dione
4-hydroxy-2,6,6-trimethyl-3-oxocyclohexene-1-carbaldehyde
4-oxoisophorone
linalool oxide (pyranoid)
linalool oxide (furanoid)
2-phenylacetaldehyde
linalool oxide (pyranoid) ketone
α-phellandrene
p-cymene
linalool
oct-1-en-3-ol
hexanal
octan-3-one
propylcyclopropane
benzaldehyde
3,5,5-trimethylhex-2-ene
(Z)-hex-3-en-1-ol
unknown benzenoid
heptane-2,3-dione
(E)-hex-3-enal
(E)-hex-2-enal
methyl 2-aminobenzoate
indole
DMNT

Appendix 2.S3

Differences in floral scent composition of evening samples of *Schiedea kaalae*, *S. hookeri*, and reciprocal hybrids, visualized by NMDS of Bray-Curtis dissimilarities between square-root transformed relative emission rates (stress = 0.11). Samples are connected by lines if collected from the same sampling bag at the same time (technical replicates), the same inflorescence on different dates, the same plankton different dates, different individuals of the same genotype (produced by propagation), or the same cross, defined by exact parentage. Volatiles that occured in more than 20% of samples are labeled at their weighted position in the ordination.



CHAPTER 3: Sexual dimorphism, diel variation, and evolutionary divergence in floral volatiles of wind-pollinated *Schiedea globosa* (Caryophyllaceae)

John M. Powers, Ann K. Sakai, Stephen G. Weller, Diane R. Campbell

Abstract

Premise

Recent evolution of separate sexes from hermaphroditism and wind pollination from biotic pollination may affect how floral scent varies over the course of the day, between sexes, and across reproductively isolated populations. We characterized this intraspecific chemical diversity in subdioecious *Schiedea globosa* (Caryophyllaceae) and compared it with genetic differences that accumulated during its radiation across the Hawaiian Islands.

Methods

Floral volatiles from inflorescences of plants grown in a common greenhouse environment (females, males, and hermaphrodites) from twelve populations (four islands) were sampled day and night and analyzed by gas chromatography – mass spectrometry (GC-MS). Differences among groups were identified by canonical analysis of principal coordinates. Relationships between scent dissimilarity and geographic or genetic distance among populations were examined by Mantel tests.

Key Results

Scent emissions changed from day to night across all sexes and populations. At night flowers increased total emission rates through higher emissions of several ketones, nitrogen-bearing oximes, and phenylacetaldehyde. Females emitted less total scent per flower but more of some aliphatic compounds than males, and males emitted more ketones and aldoximes than females. Scent differed quantitatively among populations during both day and night. Divergence in scent

increased with genetic distance for males during the day and night, but not females. Divergence in scent increased with geographic distance within 70 - 100 km for males and females at night.

Conclusions

Schiedea globosa exhibits diel and sex variation in floral scent despite wind pollination and presumed loss of biotic pollination. Surprisingly, few of the night volatiles are shared with two moth-pollinated relatives, so their production is likely not vestigial. Scent evolved with the dispersal of this species across and within islands, and in males interpopulation scent differences in a common environment were correlated with genetic differences.

Introduction

Population-level processes governing the evolution of floral scent in wind-pollinated plants, which often have separate sexes and are not constrained to signal to a pollinator by production of volatiles, may differ from those of plants with biotic pollination. However, the differences or similarities in scent blends produced by wind-pollinated flowers within populations, and across populations, sex, and times of day, have received little attention. We tested hypotheses for how scent may differ across these scales in a wind-pollinated plant species that evolved from moth-pollinated ancestors and dispersed across multiple islands.

Pollinator limitation can spur the evolution of wind pollination in flowering plants (Culley et al., 2002, Friedman and Barrett 2009) and this typically leads to weakened selection for communication with biotic pollinators and the subsequent loss of visual and chemical attractants as they become vestigial (Farré-Armengol et al., 2015, Welsford et al., 2016, Wang et al., 2019). Reductions in scent emissions in the absence of biotic pollination can be up to two orders in magnitude (Doubleday et al., 2013, Farré-Armengol et al., 2015, Sas et al., 2016), highlighting

the potential physiological or ecological costs to scent production (Theis and Adler, 2012, Kessler et al., 2013). In addition to a decrease in total emission rate compared to insect-pollinated species, volatile blends produced by wind-pollinated species may produce less of an antennal response in pollinators (Wang et al., 2019), and contain lower chemical diversity (Farré-Armengol et al., 2015, Welsford et al., 2016).

Certain conditions, however, could prevent a loss of floral scent in wind-pollinated species. First, floral scent may have roles besides attraction of pollinators, including deterrence of antagonists such as microbial pathogens, herbivores, florivores, and nectar or pollen larcenists (Schiestl, 2010, Galen et al., 2011, Delle-Vedove et al., 2017), which may prevent the loss of any volatiles with defensive functions. The release from selection for insect attraction may promote the evolution of new defensive volatiles (Dobson and Bergström, 2000). Second, if selection against the production of attractants is weak because their emission is restricted to certain times of day (Theis et al., 2007), thereby lowering their ecological or energetic costs (Baldwin et al., 1997), species that recently evolved wind pollination from biotic pollination may retain these ancestral daily changes in floral scent. For example, while *Silene dioica* is diurally pollinated, it emits moth attractants (lilac aldehydes) at night, presumably a temporal pattern retained from a moth-pollinated ancestor (Waelti et al., 2008). Daily floral scent variation may also be retained in wind-pollinated species if it serves a role in defense against diurnal or nocturnal plant antagonists (Euler and Baldwin, 1996) or is related to photosynthesis or temperature (Farré-Armengol et al., 2014). Third, as plant populations colonize areas with low pollinator abundance (Friedman and Barrett, 2009), there may be selection for increased scent signalling to attract rare pollinators prior to the transition to wind pollination. Last, plants in windy habitats

may experience dilution of their floral scent plumes, so volatile emissions can increase without increasing olfactory cues to antagonists.

Floral scent can vary among geographically separated populations as well as among species, and understanding intraspecific patterns can serve as a bridge between studies of traits within populations and studies of divergence among species across a phylogeny. All studies of intraspecific geographical variation in floral scent to date have focused on biotic pollination, and the appearance and maintenance of population differences have often been interpreted through the lens of local pollinator-mediated selection (e.g., Chapurlat et al., 2018, Dormont et al., 2019). In wind-pollinated species, genetic drift and gene flow may be the primary drivers of floral scent divergence instead of selection, which is expected to be weak because scent is not constrained to signal to a pollinator. Studies of the association of scent divergence with genetic distance between populations may address how and when scent changed during population divergence as a result of dispersal, selection, gene flow, and/or drift. However, the relationship between scent differentiation and genetic distance has been examined only for *Orphys* orchids that are biotically pollinated (Mant et al., 2005, Stökl et al., 2008). Scent and genetic distances were not correlated in *Orphrys*, which might result from strong divergent pollinator-mediated selection on scent with little genetic differentiation, rather than genetic drift.

On island archipelagos, strong barriers to gene flow between and within islands paired with selection resulting from habitat differentiation can lead to spectacular diversification. Species with multi-island distributions may experience genetic bottlenecks during colonization of new sites and islands followed by low interpopulation migration rates, which often leads to genetic and phenotypic differentiation (Spurgin et al., 2014). Examining interpopulation variation of scent in wind-pollinated plants on oceanic islands may help reveal these neutral genetic

processes, or a geographic mosaic of selection (Delle-Vedove et al., 2017) that for a wind-pollinated species is not mediated by pollinators. We expect these processes to lead to a pattern of increasing scent differentiation with genetic distance, a relationship commonly found for other floral traits across isolated populations (e.g. Leles et al., 2015, but see Pérez-Barrales et al., 2009, where no relationship between floral morphology and genetic distances was found). If reproductive barriers and colonization frequency are proportional to geographic distance, then scent differentiation would roughly follow geographic distance as well, except in cases of relatively recent long-range dispersal.

The majority of dioecious species tested to date (nearly all of them biotically pollinated) show sexual dimorphism in some aspect(s) of floral scent (compound diversity, total scent emissions, relative composition, or emission rates of particular volatiles), with males generally emitting volatiles at higher rates than females, perhaps due to mate limitation (reviewed in Ashman, 2009). There are fewer species studied for sexual dimorphism in scent that employ ambophily (8 *Salix* species, with both wind and insect pollination), or wind pollination only (9 species in 4 genera; Pellmyr et al., 1991, Dobson and Bergström, 2000, Welsford et al. 2016, Wang et al., 2019). Increased sexual dimorphism in scent is predicted in wind-pollinated taxa because males and females are no longer constrained to signal to a pollinator similarly, and so may diverge in their scent profiles (Welsford et al., 2016). In addition, females that produce costly seeds are predicted to emit more florivore repellants than males (Ashman, 2009). Sexual dimorphism may also arise from differences in the scent of floral organs that differ in size or presence between the sexes (allometry; Ashman, 2009). Alternatively, the evolution of sexual dimorphism in scent could be precluded by between-sex genetic correlations (Ashman, 2009).

Schiedea is a model genus for island evolution, with 34 species (32 extant) endemic to the Hawaiian Islands that display a range of breeding systems (Weller et al., 1998, Sakai et al., 2006), pollination modes, growth forms, and habitat preferences (Wagner et al., 2005). Subdioecious *Schiedea globosa* provides an opportunity to examine how temporal variation, sexual dimorphism, and evolutionary divergence in floral scent have been shaped by allopatric divergence and the recent evolution of wind pollination and dioecy from biotic pollination and hermaphroditism. We investigated the following questions to assess patterns and sources of this trait variation in *S. globosa*: 1) Are there daily temporal changes in floral scent, and are these similar to those of the moth-pollinated relatives of *S. globosa*? 2) Does floral scent differ between females and males, during the day or night? 3) Do interpopulation floral scent differences (within each sex and time of day) increase with geographic and/or genetic distance, consistent with phenotypic divergence of scent in allopatry? We also examined all interactions between these three factors, including sexual dimorphism in daily scent variation, variation in dimorphism in scent production among populations, and daily variation among populations.

Study system

Schiedea (Caryophyllaceae) exhibits at least two evolutionary transitions from hermaphroditism to gynodioecy and dioecy in dry habitats, and several transitions from outcrossing hermaphroditic species to autogamy in wet habitats (Sakai et al., 2006, Willyard et al., 2011). Floral adaptations to wind dispersal including greater pollen production, reduced pollen size, reduced nectaries, and condensation of the inflorescence (Weller et al., 1998; Golonka et al., 2005). Wind pollination in *Schiedea* is thought to enable the transition to separate sexes in habitats where pollinators are scarce (Weller and Sakai 1990). *Schiedea globosa*, a subdioecious, perennial subshrub, produces very condensed inflorescences elevated above the

foliage on long internodes (Figure 3.1) and experimental studies in a wind tunnel confirmed that this species has wind-dispersed pollen (Weller et al., 1998). No daytime pollinators have been observed during dozens of hours over multiple years in surveys of populations for sex expression and seed production (Weller & Sakai, pers. comm.). The coastal cliff habitats of most populations are dry and windy, which may prevent visitation and pollination by the very small moths that pollinate other *Schiedea* species occurring in mesic forests (Weller et al., 2017). Individuals produce either pistillate (female flowers with highly reduced stamens) or staminate (male flowers with highly reduced pistils) inflorescences, with a small fraction of individuals (hermaphrodites, 0-10% in the field depending on season and population) producing mostly staminate inflorescences with a few inflorescences also producing some female and/or hermaphroditic flowers, a condition apparently controlled by both environment and genetics (Sakai and Weller, 1991). In this genus male-sterility is under nuclear control (Weller and Sakai, 1991).

Schiedea globosa is broadly distributed on all the major Hawaiian Islands except Kaua‘i (Appendix 3.S1, Appendix 3.S2), with a coastal distribution and potential to colonize new islands by oceanic rafting of plants or seeds (Wagner et al., 1995). Gene flow is estimated at less than one migrant per generation, suggesting that allopatric divergence may occur (Wallace et al., 2009). *Schiedea globosa* occurs on rocky north-facing coastal cliffs and steep slopes of windward O‘ahu, Moloka‘i, West and East Maui, Lāna‘i (historical collection), and at two locations on the northeastern coast of Hawai‘i Island (Appendix 3.S2, Wagner et al., 2005). The current islands of Moloka‘i, Lāna‘i, Kaho‘olawe, and Maui were joined together as one island, referred to as Maui Nui, for 75% of the last 1.2 myr (Price and Elliott-Fisk, 2004), which may have promoted expansion and gene flow between populations on these present-day islands (Wallace et al., 2009). *Schiedea globosa* also grows in the Ko'olau mountains of eastern O‘ahu

on mesic ridges at elevations up to 560 m, and these plants have less succulent leaves and more diffuse inflorescences than those of other populations. Genetic structure between islands is strong at some loci, and overall is consistent with progression from older to younger islands with some potential backwards colonizations (Weller et al., 1996, Filatov and Burke, 2004, Wallace et al., 2009).

Plants used for volatile sampling

Volatile emissions were measured on plants of *Schiedea globosa* grown in the University of California, Irvine greenhouse. Plants originated from 12 populations from O‘ahu, Moloka‘i, Maui, and Hawai‘i, representing the entire geographic distribution of the species (localities and sample sizes given in Appendix 3.S1). In 2019 we sampled up to 4 plants per sex per population when possible (25 females, 20 males, 6 hermaphrodites), but sampling was limited in populations with few remaining plants in the wild (for example, only one plant was ever observed at Waipi‘o Valley), and in inaccessible populations with few collections of live material. The low frequency of hermaphrodites reflects their rarity in nature. The Pu‘u Kanehoalani and Ka‘a‘awa populations occur on higher elevation ridges in the Ko‘olau Mountains of O‘ahu, and three populations are located on islets less than 1.5 km offshore of the main islands (Appendix 3.S2). Plants were grown from seeds, cuttings, or in one case (Weller & Sakai 844), the offspring of controlled intrapopulation crosses. For the three populations with only one plant with genetic data (Wallace et al., 2009), we sampled the same plant as in that previous study. Plants were potted in UC mix (1:1:1 sand, peat, and redwood fiber) with added perlite and watered as needed with dilute liquid fertilizer (Grow More; 20-20-20 NPK plus micronutrients). Fresh inflorescences with 5-140 open flowers were selected for sampling. The numbers of open flowers, closed (post-anthesis) flowers, and floral buds were recorded immediately after

sampling. The number of open flowers did not affect the scent composition (canonical analysis of principal coordinates, P = 0.20).

Floral scent sampling

Procedures for dynamic headspace sampling for GC-MS were modified from Campbell et al. (2019). Scent traps, consisting of a glass capillary tube filled with 5 mg of Tenax TA adsorbent resin (80/100 mesh, Sigma-Aldrich, St. Louis, Missouri, USA) and held with plugs of silanized quartz wool, were cleaned before initial use by heating in helium carrier gas for 5 min at 250 °C. In 2019 scent samples were collected from March - June in the greenhouse during evening and daytime sampling periods. The natural day length varied from 11.5 - 14.5 h. For the day period, samples were taken with pumping start times between 11:30 - 15:30 PST, 8.2 - 4.2 h before sunset. For the evening period, samples were taken from the same inflorescence with pumping start times between 20:00 - 23:00 PST (1.4 - 3.4 h after sunset). Dynamic headspace samples of floral volatiles were taken by enclosing inflorescences in 19 x 10 cm nylon-6 oven bags (Reynolds, USA) made with an impulse sealer (Easyway HS12, USA). Volatiles were allowed to equilibrate for 30 min at 20 - 30 °C (day) or 17 - 29 °C (evening) and pumped for 30 min through a scent trap using silicone tubing and a vacuum pump (Supelco PAS-500, Spectrex, Redwood City, California, USA) set to a pre-trap flow rate of 200 mL/min. Ambient controls (n = 26) were taken from an empty oven bag sampled for the same duration. Samples were stored in capped glass vials at -20 °C until analysis. In 2018, another set of floral scent samples was taken from 38 plants (7 in common with the plants sampled in 2019) during the daytime period only (11:30 - 13:30 PST, 8.5 - 6.4 h before sunset) at temperatures from 31 - 35 °C from July - August. We analyzed these samples separately (Appendix 3.S3) because the samples were collected at higher temperatures and later in the year.

GC-MS analysis

Floral scent composition (the identity and emission rate of each volatile in the overall scent blend) was characterized and quantified by thermal desorption gas chromatography-mass spectrometry (TD-GC-MS). We employed a Shimadzu GC-MS QP2020 at the Rocky Mountain Biological Laboratory, with a 30 m × 0.25 mm internal diameter x 0.25 μm film thickness Rtx-5MS column. Scent traps were placed in a sample tube, autoloaded into a Markes UNITY-xr thermal desorption device, purged with helium for 1 min, heated to 200°C for 5 min while re-trapping on Tenax adsorbent at 25 °C, and desorbed at 200°C for 3 min. After a 2 min hold at 40 °C, the temperature of the GC oven was ramped to 210 °C at 10 °C/min, then to 275 °C at 30 °C/min and held for 2 min. The mass spectrometer was operated in electron-impact ionization mode at 70 eV and scanned in the range 35-350 m/z.

Peak deconvolution, integration, and tentative compound identification were performed in the Automated Mass Spectral Deconvolution and Identification System (AMDIS) using the NIST 2017 mass spectral library (National Institute of Standards and Technology, USA). Filtering was performed in the R package *bouquet* (github.com/jmpowers/bouquet, Eisen et al., in prep). Components were included if they had retention times between 2 and 17 min, had mass spectral match scores greater than 80%, had maximum abundances across samples greater than 4 million counts (6.5% of the median sample), and occurred in more than 3% of samples. We excluded compounds in floral samples whose mean did not exceed four times the mean in ambient samples or failed a t-test between floral samples and ambient samples with alpha adjusted to a false discovery rate of 5%. After calibration with a C7-C30 alkane ladder (Sigma-Aldrich), compound identities were verified by comparing retention indices (RI) with those given in the NIST library. Volatile emission rates were calculated within each compound

class (aliphatics, benzenoids, monoterpenes, sesquiterpenes, or nitrogen compounds) from peak integrations by calibration across 4 orders of magnitude with 2-4 replicates of 7 authentic standards ((Z)-hex-3-en-1-ol, α-pinene, indole, linalool, β-caryophyllene, benzaldehyde, (E,E)-farnesol) in methanol applied to scent traps. Emission rates were standardized by the number of open flowers and the duration of sampling (1 h total equilibration and pumping time). This scent collection and analysis method has been demonstrated to be quantitative (Bischoff et al., 2014, Campbell et al., 2019).

Scent differences among groups

To test for differences in the total scent emission rates per flower among the combinations of sex and time of day, we used a linear mixed model with plant included as a random effect and time as a fixed effect to capture the paired nature of the day and night samples. To test time effects within each sex, and compare day or night measurements across sexes for males and females, post-hoc tests were carried out using the *glht* function of the R package *multcomp* to adjust for multiple comparisons with the false discovery rate set to 5% (Hothorn et al., 2008).

To identify volatile blends that differed among groups, we employed canonical analysis of principal coordinates (CAP, Anderson and Willis, 2003, Campbell et al., 2016) with Bray-Curtis distances between scent compositions (emissions of each compound relative to total emissions), as implemented in the function *capscale* from the R package *vegan* (R Core Team, 2018, Oksanen et al., 2019). This constrained ordination method is suited to discover multivariate patterns among predefined predictors, in this case, population, sex, and time of day, and their interactions. We used a permutation test (*anova.cca*) to test each term of the full model sequentially and determine whether there were significant interactions after accounting for the main effects. To aid visual interpretation, CAP was repeated a) with only sex and time of

day as predictors and b) for each time of day with sex and population as predictors. Data were square-root transformed to reduce skew before CAP analysis.

Genetic distance analyses

We used DNA sequences from an earlier study of 59 *Schiedea globosa* individuals (1-10 individuals per population) from 10 of the 12 sampled populations to calculate genetic distances among populations (Wallace et al., 2009). We retrieved the original sequence alignments for one intergenic chloroplast locus (*psbM-trnD*) and two nuclear loci (*ncpGS* and *pepC*; all estimated to be present in single or low copy; GenBank accessions FJ496357 - FJ496647; total of 2031 bp in length). The chloroplast locus, unlike the nuclear loci, shows strong interisland genetic structure (F_{CT} = 0.79 for *psbM-trnD* vs. 0.19 for *ncpGS* and 0.03 for *pepC*), probably due to its maternal inheritance and lack of recombination (Wallace et al., 2009).

For each locus, we calculated GENPOFAD genetic distances (Joly et al., 2015) among individuals to leverage information contained in the allelic diversity within heterozygous individuals. The genetic distances were averaged across the three loci after dividing by the maximum distance at each locus (Joly and Bruneau, 2006) so that each locus was equally weighted regardless of rate of sequence evolution. This procedure generated the same composite genetic distances used by Wallace et al. (2009). We inferred a phylogeny from these distances to confirm their results using a neighbor-joining method (detailed methods and results in Appendix 3.S2).

To examine the relationship between genetic and geographic distance, Mantel tests were conducted for all populations and when populations with only one genetic sample were excluded (Weller & Sakai 964 from O‘ahu, two Hawai‘i Island populations).

To test for correlations between scent and genetic divergence at the population level, we calculated the mean genetic distance and mean Bray-Curtis scent composition (emissions of each compound relative to total emissions) distance between individuals of each population pair. A Mantel test was performed separately for each combination of sex and time of day, excluding hermaphrodites due to the lower sample size. For this analysis, the ridgetop populations in the Ko'olau Mountains of O‘ahu (Wood 13886, Wood 13885, Weller & Sakai 964) that occur within 3.4 km of each other were treated as a single population because genetic data were only available for the Weller & Sakai 964 collection.

Spatial analyses

To test for linear correlations between scent divergence and geographic distance, a Mantel test was performed on mean Bray-Curtis scent composition distance and geographic distance among populations for each combination of sex and time of day, excluding hermaphrodites. In addition to performing Mantel tests, which only detect linear correlations, we performed a loess fit for scent distance against geographic distance to detect nonlinear trends that would be expected if isolation by distance varied across scales. For each combination of sex, the scent variation within populations was plotted relative to the variation among populations.

Results

We present a summary of the detected floral volatiles and then examine how their emissions varied across time, sex, and phylogeography.

Overview of emitted volatiles

We detected 45 volatiles produced by *Schiedea globosa* in > 10% of samples, including 23 aliphatics, 3 benzenoids, 11 nitrogen compounds, 6 monoterpenes, 1 sesquiterpene, and 1

homoterpene (Table 3.S1). Including volatiles that occurred in > 3% of samples, a total of 64 volatiles were detected and used for analyses. Volatiles making up ≥ 3 % of mean emissions were, in order of mean emission rate across all samples: acetoin, (E)-3-methylbutyraldoxime, butane-2,3-dione, (Z)-3-methylbutyraldoxime, (3E)-4,8-dimethylnona-1,3,7-triene (DMNT), 1-nitropentane, phenylacetaldehyde, linalool (enantiomer unknown), and (Z)-isobutyraldoxime. To the human nose, the aldoximes lend an "old socks" smell, acetoin and butane-2,3-dione are "buttery", and phenylacetaldehyde is "sweet" (Jürgens et al., 2012, The Good Scents Company).

Emission rates of some compounds are highly correlated across samples, notably among many nitrogen compounds, the ketones acetoin and butane-2,3-dione, and the terpenes DMNT and linalool (Appendix 3.S4). The nitrogen-containing aldoximes and phenylacetaldehyde are synthesized from amino acids (Clark et al., 2009, Sørensen et al., 2018). The isomeric ratio of 3-methylbutyraldoxime was consistently 0.68 ± 0.05 E/Z (mean ± SD) when both stereoisomers were detected, which is lower than the ratios (> 1 E/Z) found in species pollinated by hawkmoths (Nielsen and Møller, 2015). The highly correlated diketones acetoin and butane-2,3-dione are typically both produced by microbial sugar fermentation, but are also reported as dominant floral volatiles in flowers that mimic fermenting fruit (Goodrich et al., 2006, Jürgens et al., 2010).

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were detected, which is lower than the ratios (> 1 E/Z) found in species pollinated by hawkmoths (Nielsen and Møller, 2015). The highly correlated diketones acetoin and butane-2,3-dione are typically both produced by microbial sugar fermentation, but are also reported as dominant floral volatiles in flowers that mimic fermenting fruit (Goodrich et al., 2006, Jürgens et al., 2010).Temporal variation in scent

Total floral scent emissions per flower were higher at night than during the day on average (Figure 3.2, Table 3.2). In females the rise in mean emissions from day to night was 60%, in males 70%, and in hermaphrodites 51%. The greatest source of multivariate variation in scent composition was due to time of day (Table 3.3); the scent change from day to night did not depend on sex, population, or sex and population combined ($p > 0.2$ for all interaction terms for time of day, Table 3.3). In addition to an overall effect of time of day, the paired samples of the same inflorescence changed in scent composition from day to night, though not always in a consistent manner (lines in Figure 3.3A).

The volatiles with the highest emission rates increased in absolute emissions from day to night but other volatiles decreased in absolute emissions (diel ratios in Appendix 3.S1). From day to night, nitrogen compounds (including (E/Z)-3-methylbutyraldoxime, 1-nitropentane, and (Z)-isobutyraldoxime) generally increased, terpenes (including DMNT and linalool) generally decreased, and benzenoids and aliphatics had varying changes (phenylacetaldehyde, acetoin, and butane-2,3-dione increased at night; Figure 3.3B; Appendix 3.S1). Diel ratios for volatiles with low emission rates or that occurred rarely (Appendix 3.S1) are subject to more technical noise, and should be interpreted with caution.

Sexual dimorphism in scent

Males produced more total scent emissions per flower than females: 53% more during the day (although $p = 0.1$) and 64% more at night (Table 3.2, Figure 3.2). The only compounds that had higher absolute emissions per flower in females than males during the day and night (of those that occurred in > 20% of all samples) were hexyl acetate (an ester with a sweet fruity odor to the human nose), 4-methylpentan-1-ol, and 4-methylhexan-1-ol (Appendix 3.S1). The large majority of compounds were emitted at higher rates in males than females during both times of day (Appendix 3.S1). Given the lower sample size of hermaphrodites, we did not formally compare them to males or females. The total scent emissions of hermaphrodites were in the same range as the other sexes (Figure 3.2) and their centroid in the CAP ordination of scent composition with sex and time predictors fell between females and males (Figure 3.3B). In the overall CAP model of scent composition with all predictors and their interactions, there was no significant interaction between sex and time but the significant sex by population interaction indicated that the scent composition difference between males and females varied among populations (Table 3.3, Figure 3.3C, 3D). The sex differences in total scent emissions per flower also varied among populations (sex by population interaction, Table 3.2). In the 2018 daytime samples, we detected a difference in scent composition between sexes, but not a difference in total scent emissions between sexes (Appendix 3.S3).

Population differences in scent

Scent composition varied among populations during the day and night (Table 3.3, Figures 3C, 3D), with the most pronounced differences between the populations on the southeast coast of O‘ahu (Weller & Sakai 844 and 906) and the rest of the populations: those from the high elevation ridges on northern O‘ahu (Weller & Sakai 964, Wood 13885 and 13886), Maui Nui,

and Hawai‘i Island. The major volatiles (making up ≥ 3 % of mean emissions) that differed among these two population groups at night were phenylacetaldehyde, (Z)-isobutyraldoxime, DMNT, and linalool (higher in the southeast O‘ahu populations), and (E/Z)-3-methylbutyraldoxime, acetoin, and 1-nitropentane (higher in the other populations). Other volatiles also discriminate the populations during the day and night (Figures 3C and 3D, respectively). In the 2018 daytime samples, we observed this same split between populations in scent composition (Appendix 3.S3).

Genetic structure among populations

No overall linear relationship between interpopulation genetic and geographic distance was detected (Mantel r = -0.04, p = 0.56). To visualize how inferred long-range dispersal events (Appendix 3.S2) affected the relationship of genetic and geographic distance, we examined the pairwise distances between the northern O‘ahu plant (Weller & Sakai 964) with other populations, and between the two Hawai‘i Island plants with other populations (Appendix 3.S5). There was a strong positive relationship between genetic and geographic distance when those three populations (from northern O‘ahu and Hawai‘i Island) were excluded (Mantel r = 0.87, p = 0.001). The genetic distances between the two Hawai‘i Island plants and the rest of the populations were lower than their high geographic distance would suggest (Appendix 3.S5). Genetic distances between the northern O‘ahu plant (Weller & Sakai 964) and other populations did not increase with geographic distance, because it was more genetically similar to the Maui Nui clade than to the southeast O‘ahu populations (Appendix 3.S5).

Scent divergence with genetic distance

Interpopulation divergence in scent composition increased linearly with interpopulation genetic distance for males during both day and night periods but not significantly so for females at either time of day (Table 3.4, Figure 3.4). For a given genetic distance, scent divergence was higher for females than males at either time of day (Figure 3.4).

Scent divergence with geographic distance

Mean scent composition distances among populations had a complex and apparently nonlinear relationship with geographic distance (Figure 3.5). We did not detect a linear relationship between these distances for any sex and time combination (Mantel tests, Table 3.4). However, in both males and females during both time periods, scent distance increased with geographic distance within historic islands, but the patterns were more variable between islands (Figure 3.5). The mean scent composition distances between nearby populations (within 25 km) was comparable to the intrapopulation distances in scent composition, for each combination of sex and time (Figure 3.5).

Within populations, the scent distances between individuals were higher for females than males and higher for day versus night samples (Figure 3.5; type III ANOVA: sex $F_{1,86} = 10.4$, $p = 0.002$; time $F_{1,86} = 5.1$, $p = 0.026$).

Discussion

Overview

In contrast to most wind-pollinated species, *Schiedea globosa* emits a strong floral scent that intensifies at night, though not with the same set of volatiles as moth-pollinated relatives

(Powers et al., 2020). All sexes produced a similar set of aliphatic, nitrogenous, and terpenoid compounds that are known to mediate diverse biotic interactions, but emissions of some compounds varied quantitatively between sexes. Scent divergence followed the intra- and inter-island colonization history of the populations revealed by genetic analyses more strongly than by geographic distance. We discuss selective and nonselective factors that could drive these levels of variation and discuss the potential functions of these compounds.

Temporal variation in scent

Strong changes of floral scent in *Schiedea globosa* were driven by the rise and fall of individual volatiles between day and night; and the increase in total scent emissions at night characteristic of moth-pollinated species was unexpected in *S. globosa* given the adaptations for wind pollination. A diverse set of volatiles including diketones, phenylacetaldehyde, and nitrogen-bearing aldoximes rose 2 - 4 times in their emissions from day to night (Table 3.3), while over the same time period, other volatiles fell to approximately half their daytime emissions (terpenes including DMNT, linalool, linalool oxides, and bergamotene). While there are no known native pollinators or herbivores of *S. globosa*, the time-specific emissions of these volatiles may have mediated biotic interactions (attraction or defense) in the deep past (as the species split from others in the genus, Willyard et al., 2011) or recent past (before humans began to transform Hawai'ian ecosystems c. 800 ya, resulting in the loss of native insects). The volatiles may also serve roles unrelated to biotic interactions. Comparisons to the functions of volatiles in other taxa may be informative but functions can be context-dependent. For example, 3-methylbutyraldoxime (emitted at higher levels at night in *S. globosa*) attracts moths to Darwin's orchid, *Angraecum sesquipedale,* and 13 other orchids (Kaiser, 1993, Nielsen and Møller, 2015) but is also emitted to attract predators of herbivores in *Oenothera* (Noge and

Tamogami, 2018). Linalool (emitted at higher levels during the day in *S. globosa*) is known as both a pollinator attractant and florivore deterrent, depending on its emission rate (Raguso 2016).

We originally hypothesized that nocturnal increases in floral scent may be a vestigial trait inherited from a moth-pollinated ancestor. In contrast, the shifts in *S. globosa* are qualitatively different from temporal scent patterns in *S. kaalae* and *S. hookeri*, relatives that occur in mesic forest and are pollinated in the evening by a moth endemic to O‘ahu (Willyard et al., 2011, Weller et al., 2017, Powers et al., 2020). These two relatives do not produce aldoximes, and have different sets of volatiles that rise at night, with the exception of phenylacetaldehyde (a general moth attractant) that rises at night in all three species and heptane-2,3-dione, which increases at night in both *S. globosa* and *S. hookeri* (the closer relative of these two species to *S. globosa*; Powers et al., 2020). The nighttime emission of the general moth attractant phenylacetaldehyde in *S. globosa* could be a vestigial trait, and the populations in southeast O‘ahu emit it at a higher rate than the other populations (Figure 3.3D). Emissions of other volatiles are shared between the three species but show distinct temporal patterns. For example, DMNT (commonly induced by leaf wounding to attract enemies of herbivores but also produced constitutively in many night-scented flowers; Tholl et al., 2011) rises at night in *S. hookeri*, but falls 20 - 60% in *S. globosa*. Similarly, three linalool oxides (hawkmoth attractants in *Clarkia breweri*; Raguso and Pichersky 1995, Raguso et al., 1996) rise at night and dominate the scent blend in *S. kaalae* but are found in less than half of *S. globosa* samples and fall 10 - 60% at night (Table 3.3).

Sexual dimorphism in scent

With the evolution of separate sexes from hermaphroditism, scent evolution in each sex will depend on evolutionary pressures and genetic or allometric controls on scent (Ashman 2009). The sexes may express different levels or kinds of volatiles if, for example, selection favored increased emission of pollinator attractants in the sex whose fertility is more limited by access to mates during the transition to wind pollination, or if females produce more defensive compounds that ward off seed predators (Ashman 2009). Some form of sexual dimorphism in scent was detected in the wind-pollinated species *Cycas rumphii* (Cycadaceae; Pellmyr et al., 1991), *Rumex acetosa* (Polygonaceae; Dobson and Bergström, 2000), and five *Leucadendron* species (Proteaceae; Welsford et al., 2016), but not in two dioecious *Thalictrum* species (Ranunculaceae; Wang et al., 2019). In *Leucadendron*, wind-pollinated species have lower floral volatiles emissions than insect-pollinated relatives but exhibit more sexual dimorphism in emissions (Welsford et al., 2016). The contrasting levels of dimorphism in floral scent of these dioecious wind-pollinated species highlight the potential for dimorphism to depend on ecological and evolutionary context.

In *Schiedea globosa*, differences in scent composition and total emissions between the sexes (females, hermaphrodites, and males) were apparent but compositional changes by sex were less pronounced than changes in daily variation (Table 3.3). Males produced 53% and 64% more emissions per flower on average than females during the day and night, respectively. Males produced relatively and absolutely higher emissions of nearly all nitrogen compounds and phenylacetaldehyde during day and night (Figure 3.3B, Appendix 3.S1). While the sample size of hermaphrodites was low, they produced a scent composition intermediate between males and females (Figure 3.3A, B) and had similar total emission rates per flower (Figure 3.2). No

differential temporal regulation of scent across sex was detected (no time by sex interaction for scent composition or total emissions; Table 3.2, Table 3.3).

The higher scent emissions per flower in males compared to females could stem from allometry (the scaling of scent emissions with the size or presence of floral organs), or differential genetic regulation. *Schiedea globosa* males have long filaments with functional anthers and short styles, while females have long styles and short filaments and small anthers that produce no viable pollen (Figure 3.1, Wagner et al., 2005). Male flowers are larger than female flowers, as judged by sepal length (males 3.0 ± 0.1 mm, females 2.4 ± 0.2 mm, mean ± SE, Golonka et al., 2005). The shaft-like nectaries, unique to the genus and the source of nectar for moths pollinating other *Schiedea* species (Harris et al., 2012, Weller et al., 2017), are more developed in males: the nectary base of *S. globosa* is 0.6-0.75 mm long in males versus 0.2-0.5 mm long in females (Wagner et al., 2005), the total nectary length is 2.0 ± 0.1 mm in males versus 1.2 ± 0.2 mm in females (mean ± SE, Golonka et al., 2005), and females produce less nectar than males, or none at all (Wagner et al., 2005). In *Schiedea*, male sterility is determined by a nuclear gene (Weller and Sakai 1991) with Mendelian inheritance that causes the anthers of females to abort late in development (Harris et al., 2012). In addition to potentially causing scent differences due to allometry of floral organs, this gene could potentially regulate synthesis of volatiles, but further work in this area is necessary.

Intraspecific variation in scent

Our prediction that interpopulation variation in floral scent increases with genetic distance was supported when comparing *S. globosa* males in different populations (for measurements taken during the day or night), but the relationship was not statistically significant for females (Table 3.4, Figure 3.4), which produce less scent and appear to have more variation in scent

composition (Figure 3.3a, Figure 3.4). Furthermore, the patterns of scent differentiation during the day and night separate two groups of populations (Figures 3C, 3D): those from southeast coast of O‘ahu (Weller & Sakai 844 and 906) and all other populations, including the ridgeline populations of northern O‘ahu (Weller & Sakai 964, Wood 13885 and 13886). The two groups differ quantitatively in the relative emissions of phenylacetaldehyde, aldoximes, aliphatics, and terpenes. The scent differences could either have arisen gradually due to selection or drift, or be caused by founder effects as seeds dispersed from southeast O‘ahu to the other locations. The populations on Maui Nui contain lower genetic diversity than those in southeast O‘ahu, presumably due to these founder effects (Weller et al., 1996). Future studies could test for changes in other floral traits, such as nectar production, between these sets of populations. Genetic drift was proposed as a mechanism for the high interpopulation variation of *Silene latifolia* in North America compared to Europe, because it was introduced to North America in small isolated populations, analogous in some ways to dispersal among islands (Dötterl et al., 2005). With genomic data, this hypothesis could be tested directly in *S. globosa*. We examined whether populations would differ in the magnitude or composition of their diel scent variation (Chapurlat et al., 2018), but did not observe such an effect (Table 3.3). The differences in scent composition and differences in total scent per flower between sexes varied among populations (Table 3.2, Table 3.3). Such geographic variation in sexual dimorphism has been reported for other reproductive and vegetative traits in wind-pollinated *Rumex hastatulus* (Puixeu et al. 2019).

Because higher gene flow is expected between nearby populations, even if they resulted from separate colonizations, we predicted that scent differentiation would be lower at shorter geographic distances. We did not detect a linear correlation between scent and geographic distance for any combination of sex and time. For females and males at night, there was a trend

of increasing scent differentiation with distance within 70 - 100 km, but beyond that distance scents became more similar between populations (Figure 3.5). In this study, populations on different historical islands (Kauaʻi, Oʻahu, Maui Nui, and Hawaiʻi) were at least 55 km away, so most of the trend of increasing night time scent differentiation with distance occurs within islands. This difference between genetic patterns and geographic patterns at large distances likely results from recent long-range dispersal events of closely related plants onto different islands (Appendix 3.S2, Appendix 3.S5). This geographic patchwork of population scent differences, not simply proportional to interpopulation geographic distance, is found in other species. In *Yucca filamentosa* and *Silene otites* (pollinated by moths and mosquitoes, respectively), no linear effect of distance on scent differentiation was found when tested on populations that were 80-1100 km apart (Svensson et al., 2005, Jhumur et al., 2008), but a positive linear effect was present for the beetle-pollinated cycad *Encephalartos villosus* at scales of 40 - 900 km, which shows an abrupt geographic break in scent chemistry within 130 km attributed to either local hybridization or pollinator-driven convergence (Suinyuy et al., 2012). While we conducted sampling in a common greenhouse environment to isolate genetic differences in scent, environmental factors can also change volatile emissions, so field studies may reveal further differentiation of scent among sites that vary in temperature, soil moisture, wind, and salinity (Majetic et al., 2009), especially between the coastal and ridgeline sites.

Conclusions

Despite morphological adaptations for wind pollination, *Schiedea globosa* inflorescences produce a strong scent that intensifies at night, although the nitrogenous and aliphatic volatiles produced at night have little overlap with those of its moth-pollinated relatives. Males produce more scent per flower than females during day and night, with some quantitative variation in the

ratios of the male and female scent blends, which could be explained by allometry in the sizes of floral organs in each sex. Populations in southeast O'ahu differ the most from other populations genetically and in scent composition. Scent differentiation was associated with genetic distance closely in males, and was associated with geographic distance weakly for females during the day. This combination of genetic and chemical analyses showed that a recent evolutionary radiation can lead to floral scent divergence in tandem with genetic divergence. Similar processes acting along longer time spans could explain floral scent divergence at larger temporal and geographic scales, including among species undergoing island radiations.

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Figures, Tables, and Appendices

Figure 3.1

(a) Habitat of *Schiedea globosa* at Kahakuloa on West Maui. (b) Pistillate (female) flowers showing long styles (reduced stamens are adjacent to the base of the ovary, marked by white triangles). (c) Staminate (male) flowers with long stamens with yellow anthers and reduced styles. The flower in the center of the male inflorescence (white triangle) has longer than usual styles; occasional hermaphrodites have even longer styles with functional stigmas on some flowers. Male flowers are larger than female flowers, as measured by sepal length (males 3.0 ± 0.1 mm, females 2.4 ± 0.2 mm, mean ± SE, Golonka et al., 2005). Photos by Stephen G. Weller.

Figure 3.2

Total volatile emission rate per flower from the three sexes during day and night. Lines connect measures of the same plant at different times. Pairwise comparisons of group means indicate the percent increase in emission rates, and p-values are adjusted by the false discovery rate method. Linear mixed model results are given in Table 3.2.

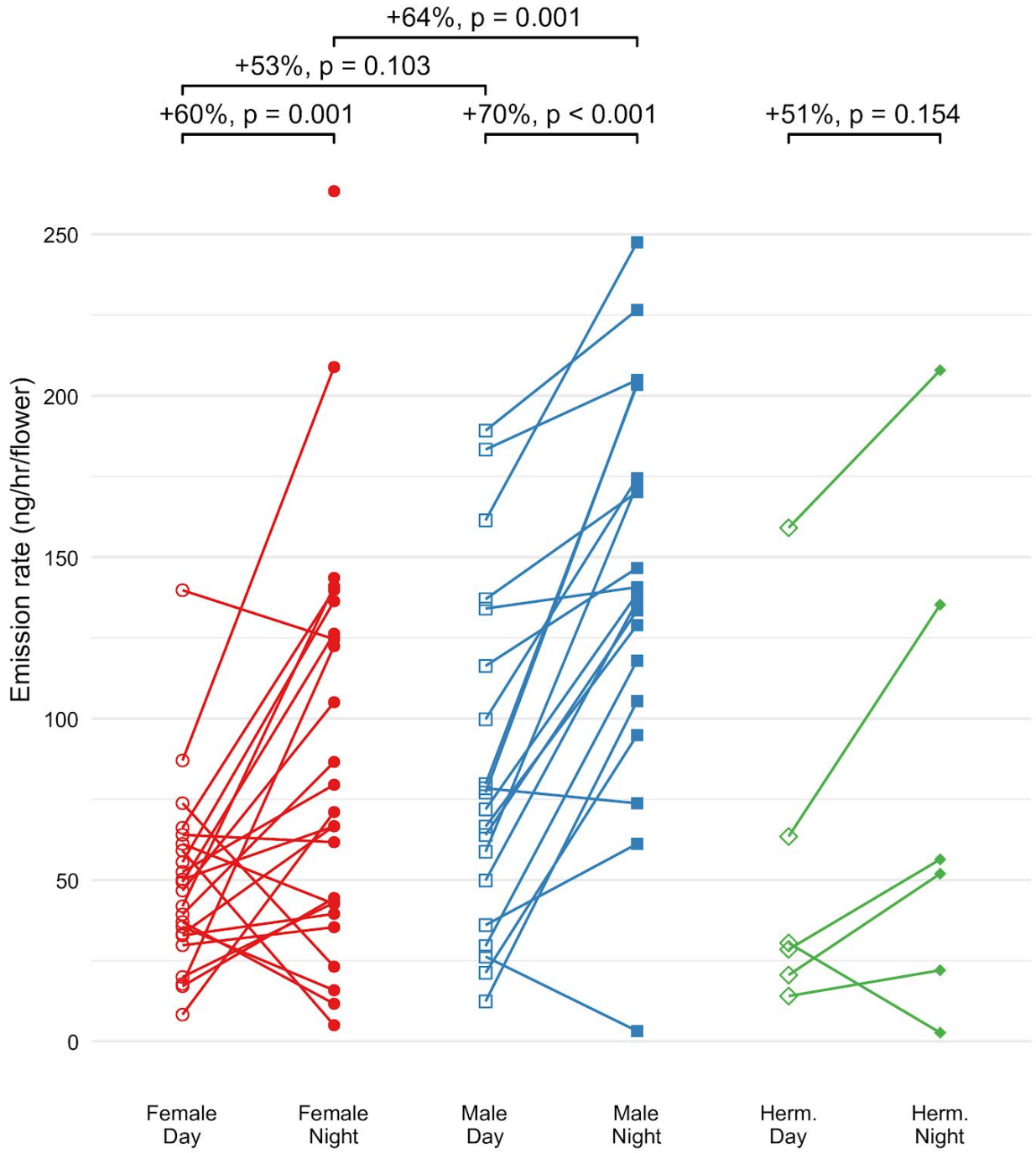
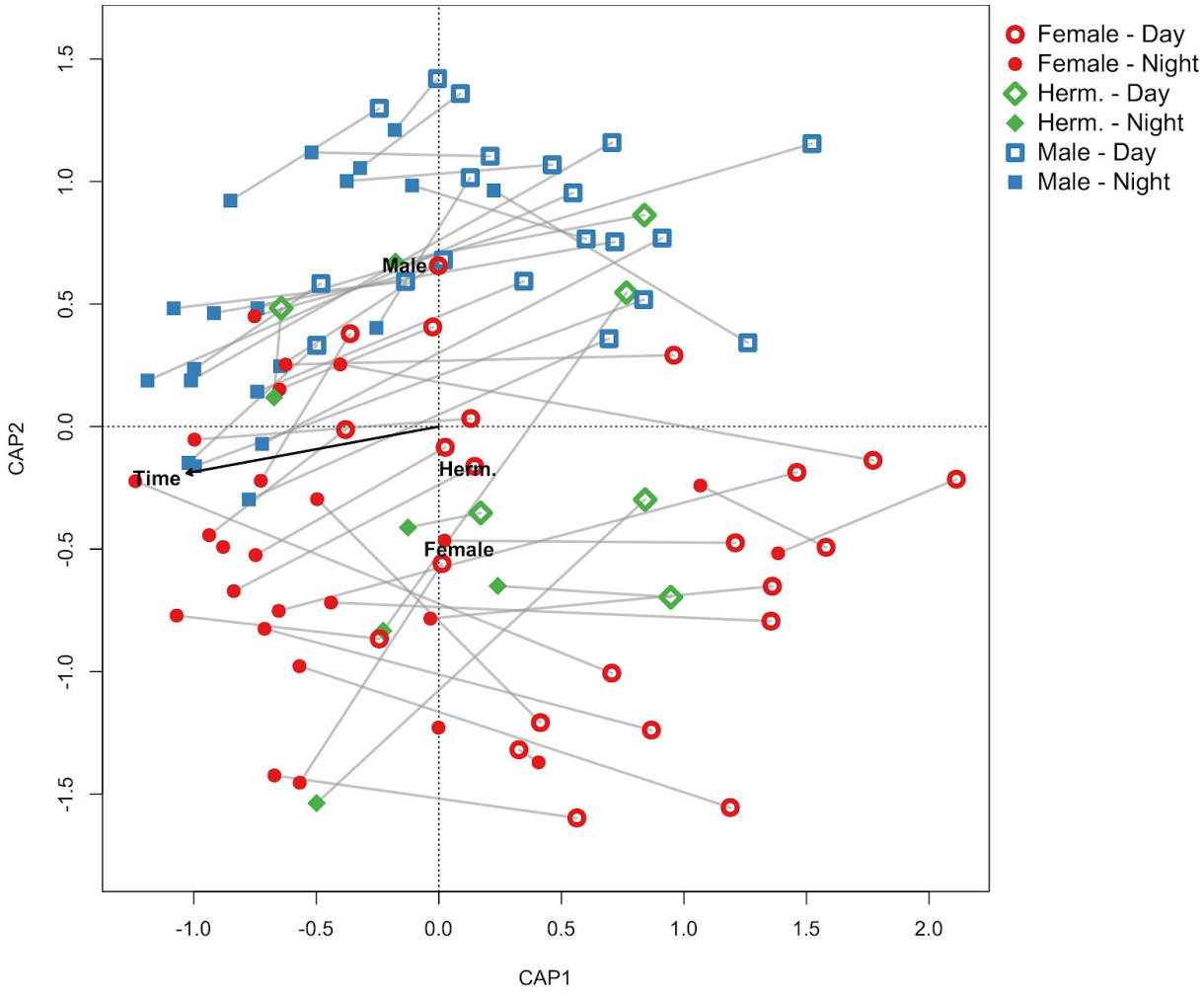


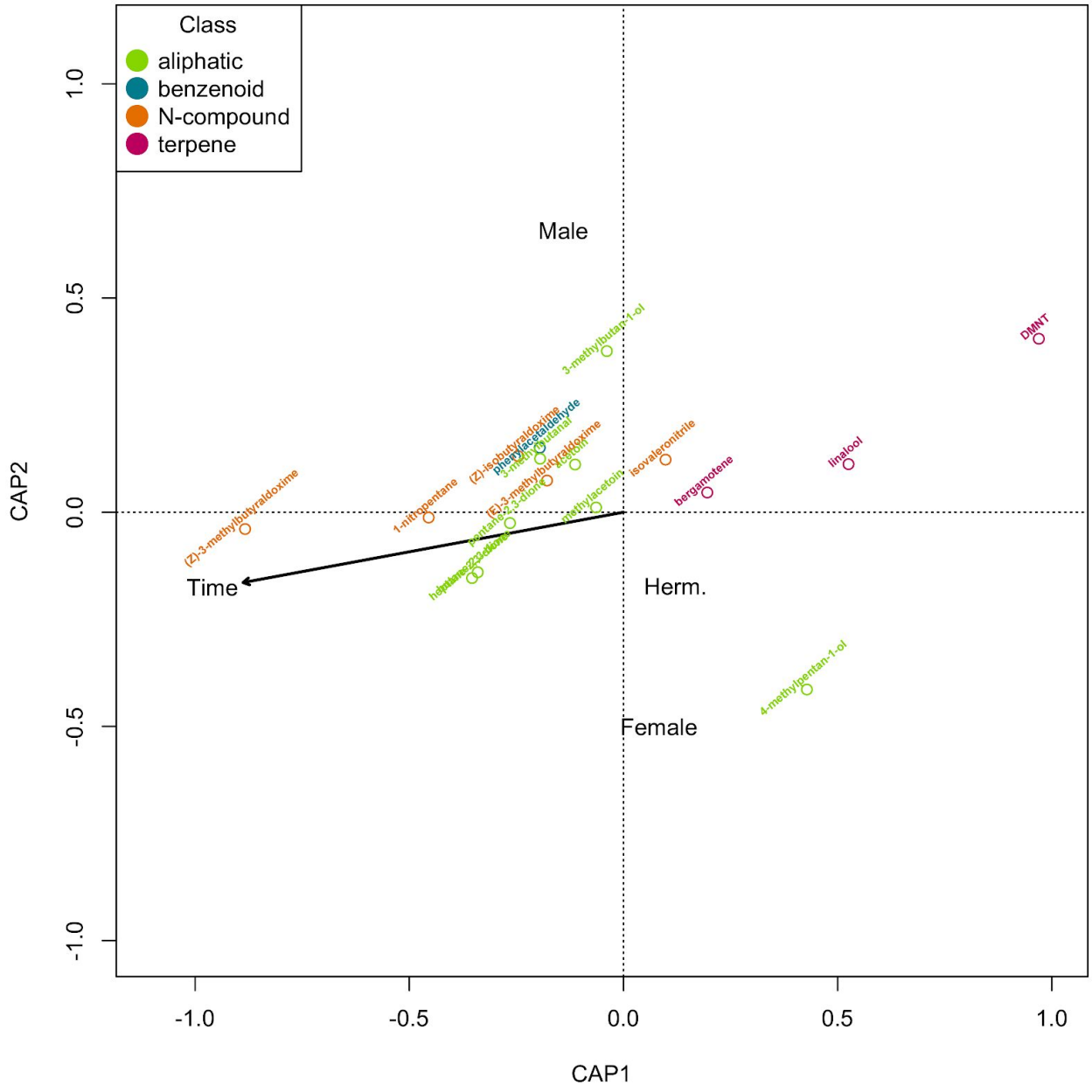
Figure 3.3

Canonical analysis of principal coordinates (CAP) separating *Schiedea globosa* floral scent samples by sex and time of day (Panels A and B) or by sex and population for each time of day (Panels C and D). The analysis used scent compositions (the emissions of each compound relative to total emissions) and Bray-Curtis distances. Sex (female, male, hermaphrodite) is indicated by shape, and time of day is indicated by hollow (day) or filled (night) shapes. Panel A shows samples connected by lines in day-night pairs. Panel B shows the loadings of the compounds (labeled circles) on the same CAP axes as Panel A, with scales expanded for visualization. In Panels A and B the centroids of samples of the three sexes are indicated by the label (male, female, hermaphrodites), and the arrow points toward a later time of sampling relative to sunset. Panels C and D show the same samples split into separate CAP analyses for day (C) and night (D), with samples from a population connected by lines, and loadings of the compounds shown as labels on the plot. Statistics for the full model are in Table 3.3.

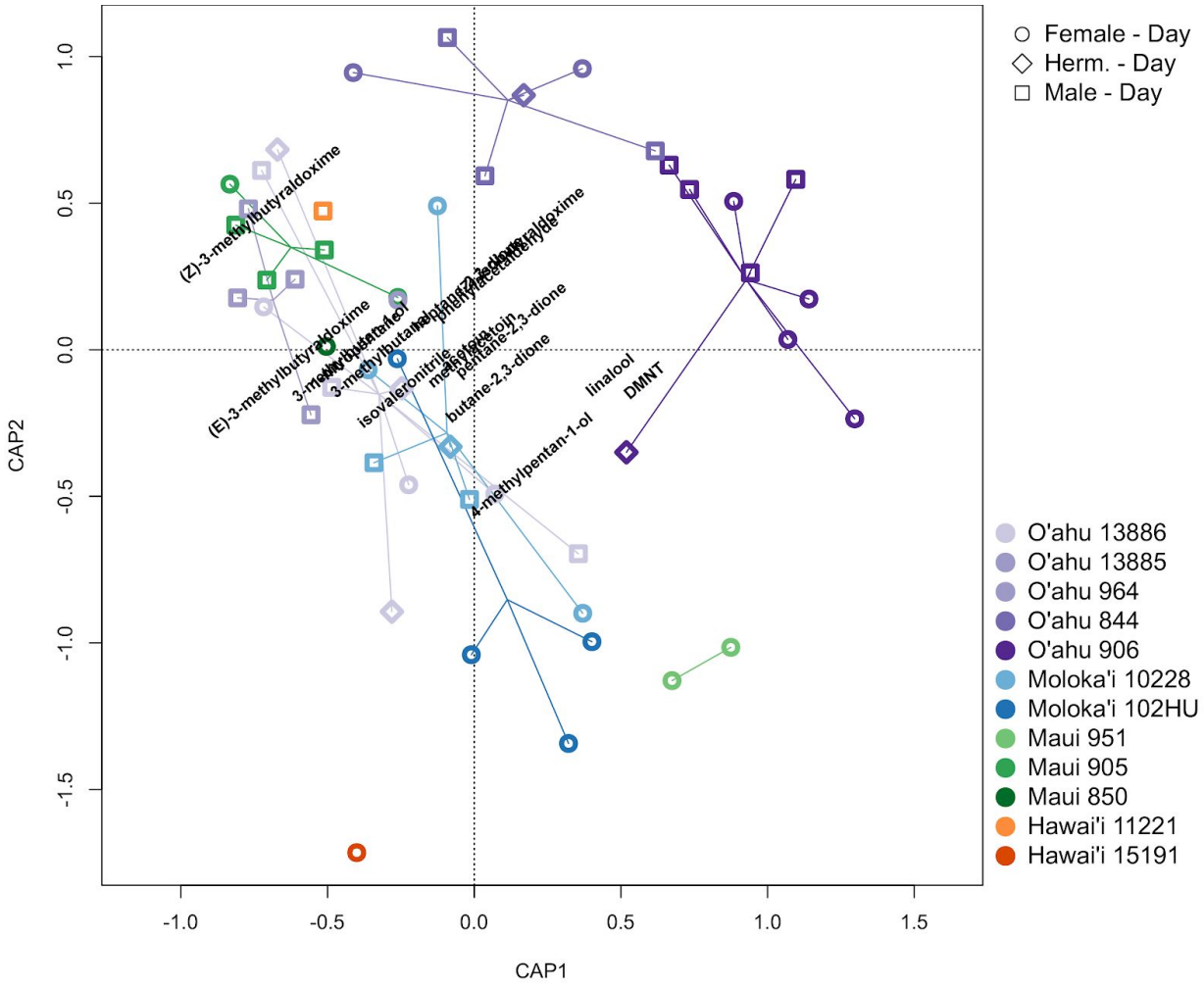
3A



3B


Class
aliphatic
benzenoid
N-compound
terpene
Male
Female
Herm.
Time
CAP1
CAP2
-1.0
-0.5
0.0
0.5
1.0
3-methylbutan-1-ol
DMNT
linalool
bergamotene
isovaleronitrile
(Z)-3-methylbutyraldoxime
1-nitropentane
(Z)-isobutyraldoxime
phenylacetaldehyde
3-methylbutyraldoxime
(E)-3-methylbutyraldoxime
methylacetoin
4-methylpentan-1-ol

3C


Female - Day
Herm. - Day
Male - Day
O'ahu 13886
O'ahu 13885
O'ahu 964
O'ahu 844
O'ahu 906
Moloka'i 10228
Moloka'i 102HU
Maui 951
Maui 905
Maui 850
Hawai'i 11221
Hawai'i 15191
(Z)-3-methylbutyraldoxime
(E)-3-methylbutyraldoxime
isovaleronitrile
butane-2,3-dione
4-methylpentan-1-ol
linalool
DMNT
CAP1
CAP2
-1.0
-0.5
0.0
0.5
1.0
1.5
-1.5
-1.0
-0.5
0.0
0.5
1.0

3D

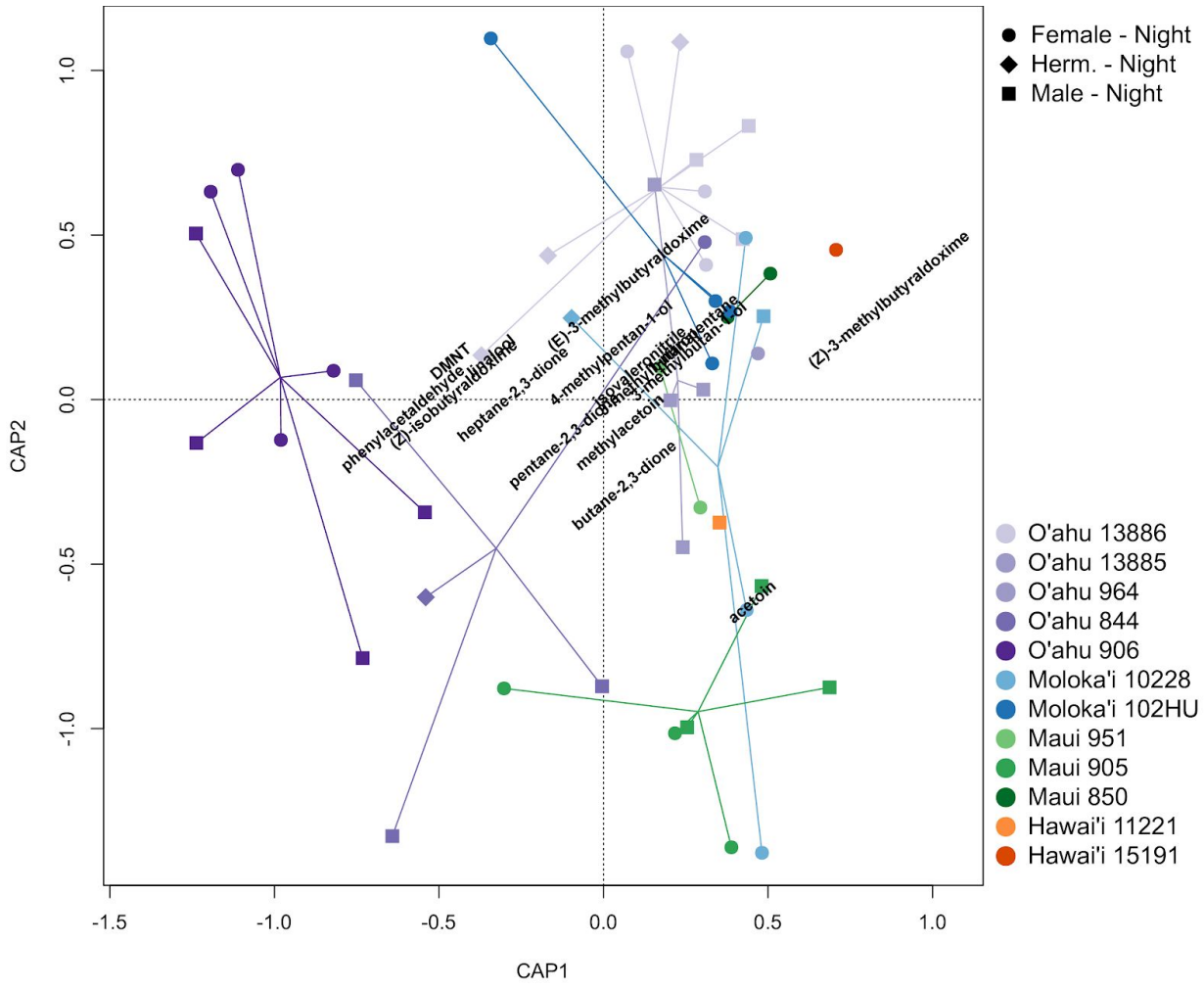

Female - Night
Herm. - Night
Male - Night
O'ahu 13886
O'ahu 13885
O'ahu 964
O'ahu 844
O'ahu 906
Moloka'i 10228
Moloka'i 102HU
Maui 951
Maui 905
Maui 850
Hawai'i 11221
Hawai'i 15191
CAP1
CAP2
-1.5
-1.0
-0.5
0.0
0.5
1.0
DMNT
phenylacetaldehyde
(Z)-isobutyraldoxime
heptane-2,3-dione
(E)-3-methylbutyraldoxime
4-methylpentan-1-ol
isovaleronitrile
pentane-2,3-dione
methylacetoin
butane-2,3-dione
acetoin
(Z)-3-methylbutyraldoxime

Figure 3.4

Plot of mean scent distance among populations versus mean genetic distance among populations for two sexes (male and female, indicated by color and shape) and two time periods (day and night, indicated by fill and line type). The pairwise distances among individuals of each sex at each time were averaged to calculate the mean interpopulation distances. The scent distance metric is the Bray-Curtis distance between individual scent compositions (the emissions of each compound relative to total emissions). The genetic distance metric is the composite (mean of three equally-weighted genes) GENPOFAD genetic distance between individuals. Linear models are fitted for each combination of sex and time. There was a statistically significant linear correlation between scent distance and genetic distance for males during both day and night, but not females (Mantel tests, Table 3.4).

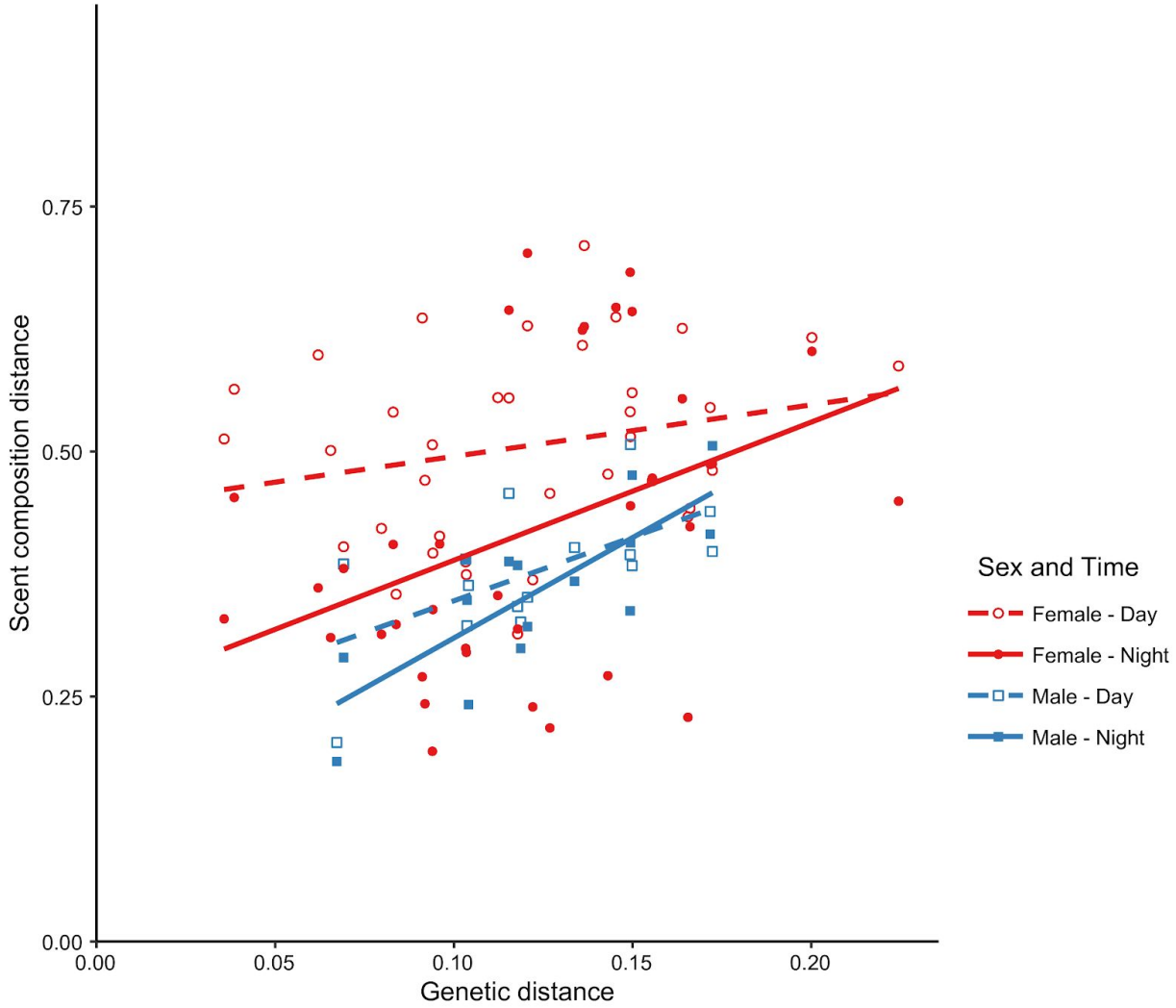


Figure 3.5

Plot of mean scent distance among populations versus geographic distance among populations for two sexes (male and female, indicated by color and shape) and two time periods (day and night, indicated by fill and line type). The pairwise distances among individuals of each sex at each time were averaged to calculate the mean interpopulation distances. The scent distance metric is the Bray-Curtis distance between individual scent compositions (the emissions of each compound relative to total emissions). Within-population scent distances are shown to the left as boxplots. Loess curves are fitted for each combination of sex and time. A vertical line at 55 km divides the pairwise distances within and between historical islands, where the current islands of Maui and Moloka‘i are considered as one island (Maui Nui). There was no statistically significant linear correlation between scent distance and geographic distance for any combination of sex or time (Mantel tests, Table 3.4).

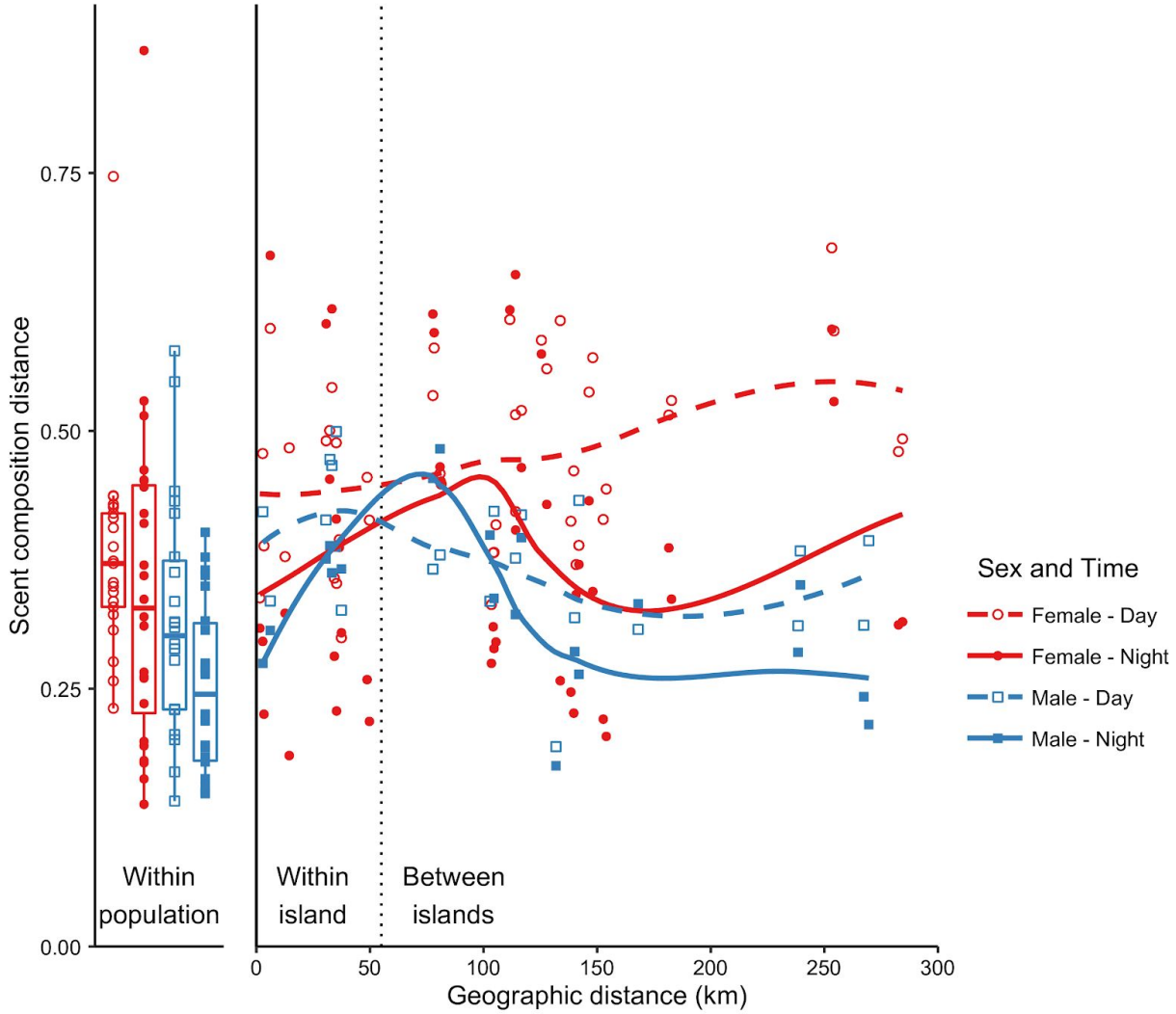


Table 3.1

Volatiles emitted from *Schiedea globosa* male and female inflorescences, during day and night periods. Compounds that occur in more than 10% of samples are shown, and the proportion of samples (Prop.) in which they occur are in bold if > 50%. The diel ratio (night / day) of emissions indicates whether the compound increased in emission at night (> 1, bolded) or decreased at night (< 1). The sex ratio (male / female) shows whether the compound was emitted at a higher rate in males than females (> 1, bolded) or not (< 1). Mean floral volatile emissions > 1 ng/flower/hr are in bold.

Class	**Compound**	**Prop.**	**Diel ratio (night/day)**		**Sex ratio (M/F)**		**Mean emission rate (ng/flower/hr)**			
			Female	**Male**	**Day**	**Night**	**F Day**	**F Night**	**M Day**	**M Night**
Aliphatic										
alcohol	4-methylpentan-1-ol	**77%**	0.9	0.4	0.4	0.1	**1.7**	**1.5**	0.6	0.2
	4-methylhexan-1-ol	**64%**	0.8	0.1	0.2	0.0	0.4	0.4	0.1	0.0
	3-methylbutan-1-ol	**58%**	**2.1**	**1.1**	**9.0**	**4.9**	0.3	0.5	**2.3**	**2.6**
	oct-1-en-3-ol	**54%**	0.8	0.6	**1.7**	**1.4**	0.2	0.2	0.4	0.2
	2,7-dimethyloctane-4,5-diol	44%	0.5	**13.7**	0.1	**1.6**	0.2	0.1	0.0	0.2
	pentan-1-ol	34%	**2.7**	0.9	**1.3**	0.4	0.1	0.2	0.1	0.1
aldehyde	3-methylbutanal	**98%**	**3.4**	**2.0**	**3.7**	**2.2**	0.6	**2.0**	**2.2**	**4.3**
	(E)-hex-2-enal	43%	0.5	**1.1**	0.9	**2.0**	0.1	0.1	0.1	0.1
	(Z)-hept-2-enal	15%	0.0	0.3	**10.9**		0.0	0.0	0.2	0.0
alkane	2,3-dimethylpentane	21%	**4.2**	0.3	**2.4**	0.2	0.0	0.1	0.1	0.0
	2,5,5-trimethylhex-2-ene	14%	**1.1**	0.9	**1.8**	**1.5**	0.0	0.0	0.0	0.0
ester	hexyl acetate	**60%**	**1.5**	0.9	0.9	0.5	0.4	0.6	0.3	0.3
	hexyl carbonochloridate	14%	0.7	0.7	0.9	0.9	0.1	0.1	0.1	0.1
	[(E)-hex-3-enyl] acetate	11%	**4.4**	0.8	**2.9**	0.6	0.2	0.8	0.5	0.5
ether	2-(methoxymethyl)oxirane	**51%**	**1.9**	**2.2**	**1.4**	**1.6**	0.1	0.1	0.1	0.2
ketone	acetoin	**100%**	**1.8**	**2.2**	**1.7**	**2.0**	**8.8**	**16.2**	**14.7**	**32.2**
	butane-2,3-dione	**99%**	**2.4**	**2.3**	**1.5**	**1.4**	**6.2**	**14.9**	**9.4**	**21.5**
	2-ethylcyclopentan-1-one	**80%**	**1.1**	0.5	**1.7**	0.9	0.2	0.2	0.4	0.2
	pentane-2,3-dione	**73%**	**3.9**	**3.9**	**1.8**	**1.8**	0.2	1.0	0.4	**1.7**
	heptane-2,3-dione	48%	0.5	**16.5**	0.0	**1.6**	**1.7**	0.9	0.1	**1.4**
	2-methylpentan-3-one	35%	0.7	**2.4**	0.3	1.0	0.0	0.0	0.0	0.0

	hexane-2,3-dione	13%	**5.4**	**2.9**	**5.6**	**3.0**	0.0	0.0	0.0	0.1
	cyclopentane-1,2-dione	12%	0.9	0.0	**59.0**	**2.7**	0.0	0.0	0.4	0.0
Benzenoid										
aldehyde	phenylacetaldehyde	**94%**	**3.0**	**4.3**	**1.8**	**2.6**	**1.0**	**3.0**	**1.9**	**8.0**
carboxylic acid	8-oxo-8-phenyloctanoic acid	24%	0.8	**9.0**	0.1	**1.1**	0.3	0.3	0.0	0.3
ether	anisole	**66%**	0.3	0.2	**1.1**	0.8	0.2	0.1	0.2	0.1
Nitrogen compound										
aldoxime	3-methylbutyraldoxime	**96%**	**2.3**	**1.6**	**2.3**	**1.6**	**5.6**	**12.7**	**12.6**	**20.1**
	isobutyraldoxime.1	**79%**	**2.8**	**2.6**	**2.2**	**2.0**	0.7	**2.1**	**1.6**	**4.2**
	2-methylbutyraldoxime	**61%**	**3.2**	**2.3**	**2.4**	**1.7**	0.0	0.1	0.1	0.2
	3-methylbutyraldoxime.1	**52%**	**4.0**	**1.8**	**2.9**	**1.3**	**3.7**	**14.7**	**10.5**	**19.5**
	isobutyraldoxime	17%	**1.7**	0.0	0.6	0.0	0.0	0.1	0.0	0.0
nitrile	isovaleronitrile	**96%**	**1.6**	**1.2**	**2.4**	**1.7**	1.0	**1.6**	**2.3**	**2.7**
	isobutyronitrile	**74%**	**1.2**	**1.4**	**1.7**	**2.0**	0.1	0.1	0.1	0.2
	2-methylbutanenitrile	37%	**3.5**	**1.6**	**7.9**	**3.5**	0.0	0.0	0.0	0.1
nitroalkane	1-nitropentane	**78%**	**3.1**	**3.0**	**1.8**	**1.7**	**1.4**	**4.5**	**2.5**	**7.6**
	2-nitropentane	34%	**30.3**	**1.9**	**38.4**	**2.4**	0.0	0.1	0.1	0.2
pyrazine	2,3,5,6-tetramethylpyrazine	20%	**1.7**	0.8	**3.1**	**1.4**	0.2	0.3	0.5	0.4
Terpene										
acyclic monoterpene alcohol	linalool	**80%**	0.6	0.6	**1.3**	**1.3**	**3.2**	**1.9**	**4.1**	**2.5**
	epoxydihydrolinalool	11%	**3.5**	0.4	**7.0**	0.9	0.0	0.0	0.1	0.0
cyclic monoterpene	a-thujene	12%	**1.1**	**4.3**	0.8	**3.2**	0.1	0.1	0.1	0.3
cyclic monoterpene alcohol	linalool oxide (pyranoid)	46%	0.9	0.9	**2.5**	**2.5**	0.1	0.1	0.4	0.3
	linalool oxide (pyranoid) ketone	43%	0.4	0.5	0.9	1.0	0.2	0.1	0.2	0.1
	linalool oxide (furanoid).2	11%	0.7	**1.1**	**1.8**	**2.8**	0.1	0.1	0.2	0.2
cyclic sesquiterpene	bergamotene	16%	0.5	0.4	1.0	0.8	0.8	0.4	0.8	0.3
homoterpene	DMNT	**92%**	0.4	0.8	**1.5**	**2.8**	**7.1**	**3.0**	**11.0**	**8.3**

Table 3.2

Results of a linear mixed model of total scent emissions per flower with predictors time of day, sex (female, male, or hermaphrodite), population, and their interactions. Plant identity was included as a random effect to model the paired day and night samples. The model was tested using type III ANOVA via Satterthwaite's degrees of freedom method. Pairwise tests comparing group means are shown in Figure 3.2.

Predictor	SS	MS	numerator df	denominator df	F	p
Time	26645	26645	1	26.2	23.54	**<0.001**
Sex	9034	4517	2	26.5	3.99	**0.031**
Population	81165	7379	11	27.2	6.52	**<0.001**
Time * Sex	4055	2028	2	25.9	1.79	0.187
Time * Population	12816	1165	11	26.5	1.03	0.449
Sex * Population	19800	2829	7	26.5	2.50	**0.041**
Time * Sex * Population	7046	1007	7	25.9	0.89	0.529

Table 3.3

Canonical analysis of principal coordinates (CAP) of floral scent compositions (the emissions of each compound relative to total emissions) using Bray-Curtis distances and tested with 999 permutations. The analysis excludes hermaphrodites because of the low sample size. The predictors explained a total of 65% of the total variation (inertia).

Predictor	df	SS	F	p
Time	1	0.916	11.86	**0.001**
Sex	1	0.284	3.68	**0.002**
Population	11	2.511	2.95	**0.001**
Time * Sex	1	0.063	0.82	0.627
Time * Population	11	0.898	1.06	0.330
Sex * Population	4	0.571	1.85	**0.003**
Time * Sex * Population	4	0.245	0.79	0.850
Residual	54	4.172		

Table 3.4

Mantel tests of scent composition against geographic or genetic distance for each subset of time of day and sex (N=10 populations for genetic distance and N=12 populations for geographic distance). The Mantel statistic r and the associated p-value are given, as well as the slope and intercept. The slope for geographic distance has units of Bray-Curtis scent distance / 100 km, and the slope for the genetic distance has units of Bray-Curtis scent distance per genetic (GENPOFAD) distance. There is a nonlinear relationship between geographic and genetic distance not captured by the Mantel tests.

		Geographic					**Genetic**			
Time	**Sex**	**r**	**p**	**slope**	**intercept**		**r**	**p**	**slope**	**intercept**
Day	Female	0.37	0.110	0.047	0.42		0.24	0.226	0.67	0.40
Night	Female	0.03	0.307	0.010	0.37		0.41	0.102	1.37	0.24
Day	Male	-0.38	0.905	-0.017	0.39		**0.60**	**0.014**	**1.44**	0.18
Night	Male	-0.47	0.913	-0.022	0.35		**0.78**	**0.001**	**1.88**	0.11

Appendix 3.S1: Plant material

S. globosa populations used in this study, with collection numbers (Wagner et al., 2005), island and locality, and sample sizes of plants of each sex. Because clones and offspring of field plants were used for sampling, the number of unique genotypes is given in parentheses.

Island	**Collection number**	**Locality**	**Female**	**Herm.**	**Male**
Oʻahu	Wood 13886	Kaʻaʻawa	3 (3)	3 (2)	3 (3)
Oʻahu	Wood 13885	Puʻu Kanehoalani			4 (2)
Oʻahu	Weller & Sakai 964	Puʻu Kanehoalani	1 (1)		
Oʻahu	Weller & Sakai 844	Makapuʻu	2 (1)	1 (1)	3 (2)
Oʻahu	Weller & Sakai 906	Koko Head	4 (2)	1 (1)	4 (2)
Molokaʻi	Wood 10228	Mokapu Islet	3 (3)	1 (1)	2 (2)
Molokaʻi	Wood 102HU	Huelo Islet	4 (3)		
West Maui	Weller & Sakai 951	Pohakupule Gulch	2 (1)		
West Maui	Weller & Sakai 905	Honokohau	3 (3)		3 (3)
West Maui	Weller & Sakai 850	Waiheʻe	2 (1)		
Hawaiʻi	Wood 11221	Paokalani Islet			1 (1)
Hawaiʻi	Perlman & Wood 15191	Waipiʻo Valley	1 (1)		

Appendix 3.S2: Phylogeography

Methods

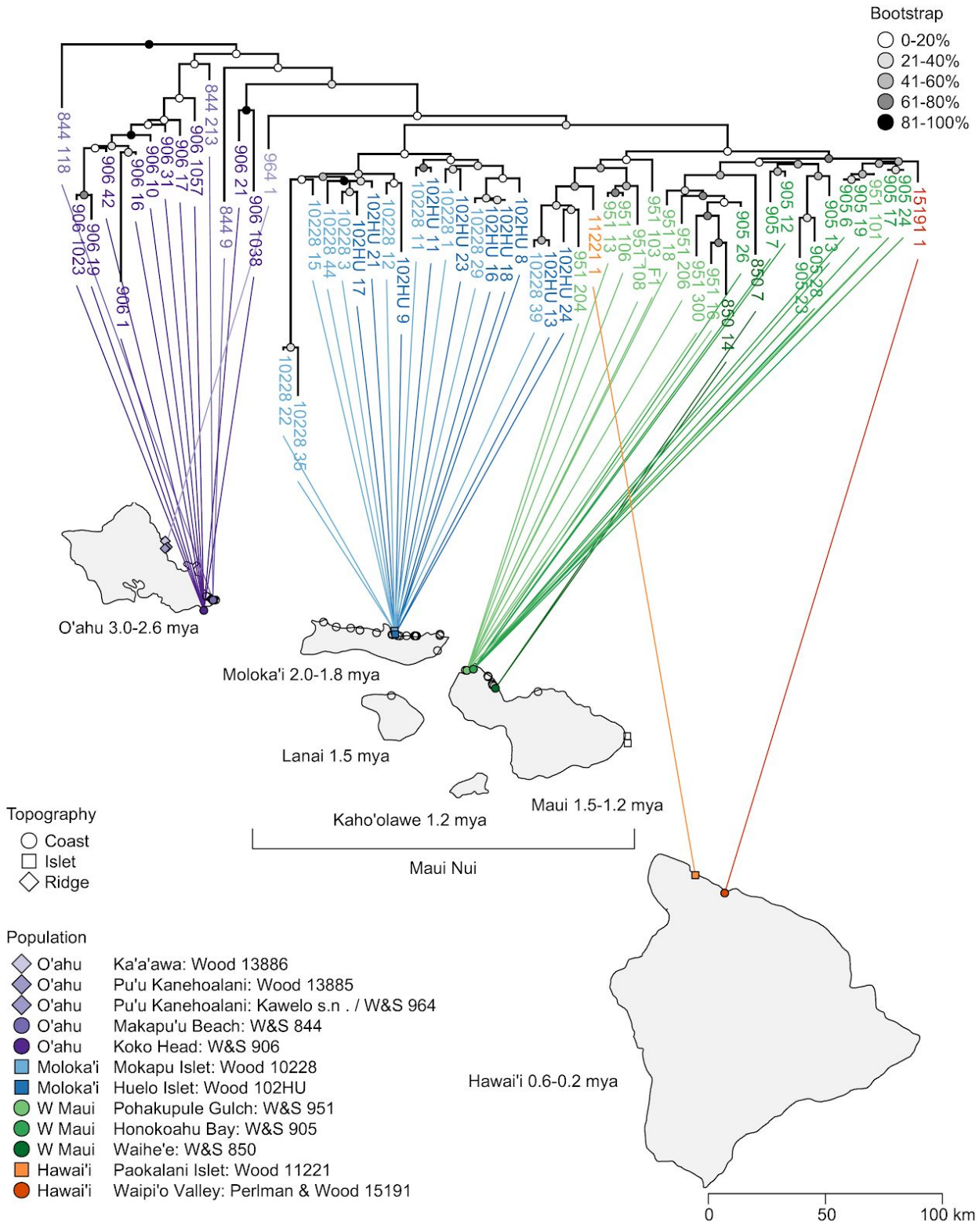
We constructed a phylogenetic tree using the neighbor-joining method (Saitou and Nei, 1987) from composite GENPOFAD distances generated from two nuclear loci (*pepC, ncpGS*) and one chloroplast locus (*psbM-trnD*) from the alignment reported in Wallace et al. (2009). The tree was rooted using four other *Schiedea* species as outgroups (Wallace et al., 2009). We implemented bootstrap support values using the procedure suggested in Shahin et al. (2014) for resampling the alignments 100 times, recalculating the composite distances, and summarizing the set of resulting bootstrap trees. To superimpose the tree on a map, the nodes of the tree were rotated to best match the order of the tips and the rank order of the longitudes of the populations using the function *phylo.to.map* in the R package *phytools* (Revell 2012).

Results

The neighbor-joining tree recapitulated the phylogeographic patterns within *S. globosa* that were reported as a NeighborNet phylogenetic network in Wallace et al. (2009). The bootstrap values indicate only weak support for relationships above the island level.The inferred patterns of colonization are consistent with populations colonizing newer islands from older islands (island progression rule), with the paraphyletic Oʻahu clade splitting first from the outgroup *Schiedea* species (not shown), the clade for Maui Nui (Molokaʻi and Maui) and northern Oʻahu nested with the Oʻahu clade, and the two Hawaiʻi Island plants nested within the Maui clade, probably representing recent colonization of Hawaiʻi Island from Maui. Populations are grouped by island with two previously reported exceptions (Wallace et al., 2009): the northern Oʻahu plant (Weller & Sakai 964) is placed sister to the Maui Nui clade, and the three individuals from Molokaʻi are nested within the Maui clade. Because incomplete lineage sorting and historical gene flow are expected in such a young radiation, this tree representation of relatedness should be treated with caution.

Phylogeny

Phylogeography of *Schiedea globosa* populations used in this study. Populations are indicated by their locality and collection number (W&S: Weller & Sakai). The phylogenetic tree was inferred using the neighbor-joining method on composite GENPOFAD distances. Bootstrap support values are indicated by the shading at the end of each branch. The ages of the islands are from Clague (1996). The current islands that make up the historical island of Maui Nui are indicated by a bracket. Island outlines are drawn from the *mapdata* R package (Brownrigg 2018). The location of the Lānaʻi population is approximate.


Bootstrap
0-20%
21-40%
41-60%
61-80%
81-100%
O'ahu 3.0-2.6 mya
Moloka'i 2.0-1.8 mya
Lanai 1.5 mya
Kaho'olawe 1.2 mya
Maui 1.5-1.2 mya
Maui Nui
Hawai'i 0.6-0.2 mya
Topography
Coast
Islet
Ridge
Population
O'ahu Ka'a'awa: Wood 13886
O'ahu Pu'u Kanehoalani: Wood 13885
O'ahu Pu'u Kanehoalani: Kawelo s.n . / W&S 964
O'ahu Makapu'u Beach: W&S 844
O'ahu Koko Head: W&S 906
Moloka'i Mokapu Islet: Wood 10228
Moloka'i Huelo Islet: Wood 102HU
W Maui Pohakupule Gulch: W&S 951
W Maui Honokoahu Bay: W&S 905
W Maui Waihe'e: W&S 850
Hawai'i Paokalani Islet: Wood 11221
Hawai'i Waipi'o Valley: Perlman & Wood 15191
0
50
100 km

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Wallace, L. E., Weller, S. G., Wagner, W. L., Sakai, A. K., and Nepokroeff, M. (2009). Phylogeographic patterns and demographic history of *Schiedea globosa* (Caryophyllaceae) on the Hawaiian Islands. *Am. J. Bot.* 96, 958–967. doi:10.3732/ajb.0800243.

Appendix 3.S3: Analyses of plants sampled in 2018

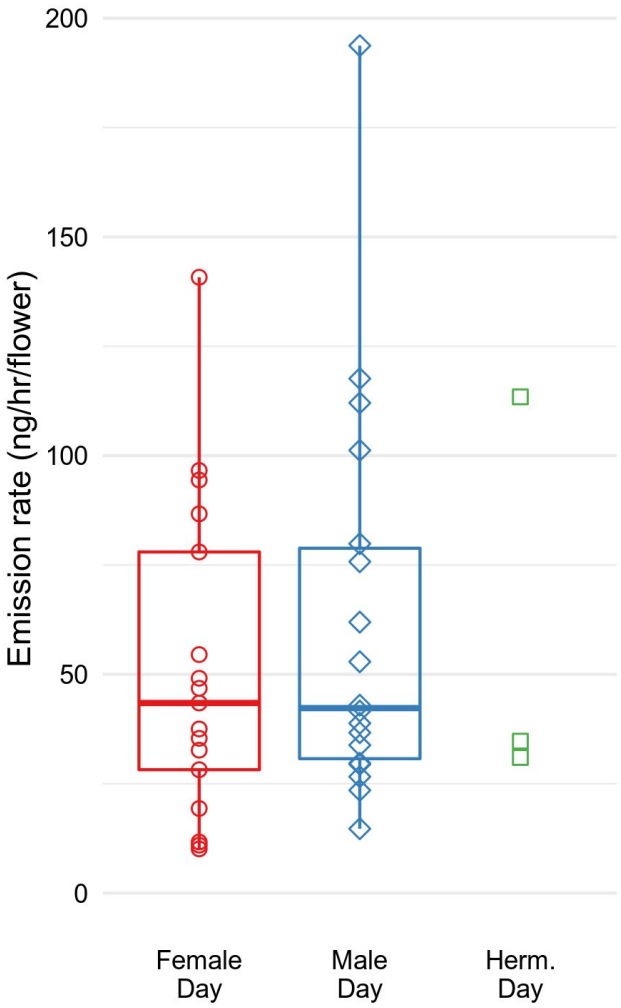
Plants sampled in 2018

S. globosa populations used in this study, with collection numbers (Wagner et al., 2005), island and locality, and sample sizes of plants of each sex sampled in 2018. Because clones and offspring of field plants were used for sampling, the number of unique genotypes is given in parentheses. These 38 plants (35 genotypes) were all sampled during the daytime period. Of these, 7 plants (7 genotypes) were sampled again in 2019. The analyses of the 2018 samples below gave similar results when these 7 plants were excluded, and the scent differences between populations detected by CAP were similar for these 7 plants in both years.

Island	**Collection number**	**Locality**	**Female**	**Herm.**	**Male**
Oʻahu	Wood 13886	Kaʻaʻawa	1 (1)	1 (1)	7 (7)
Oʻahu	Wood 13885	Puʻu Kanehoalani	1 (1)		3 (3)
Oʻahu	Weller & Sakai 964	Puʻu Kanehoalani	1 (1)		
Oʻahu	Weller & Sakai 844	Makapuʻu	4 (4)	1 (1)	4 (3)
Oahu	Weller & Sakai 906	Koko Head	3 (2)	1 (1)	3 (3)
Molokaʻi	Wood 10228	Mokapu Islet	1 (1)		1 (1)
Molokaʻi	Wood 102HU	Huelo Islet	4 (3)		
West Maui	Weller & Sakai 951	Pohakupule Gulch			
West Maui	Weller & Sakai 905	Honokohau	1 (1)		
West Maui	Weller & Sakai 850	Waiheʻe			
Hawaiʻi	Wood 11221	Paokalani Islet			
Hawaiʻi	Perlman & Wood 15191	Waipiʻo Valley	1 (1)		

Total volatile emission rate by sex

Total volatile emission rate per flower from the three sexes during the day. There was no effect of sex on total emission rate per flower (n = 38, P = 0.76). Box plots for females and males indicate the minimum, first quartile, median, third quartile, and maximum.



Analysis of scent composition

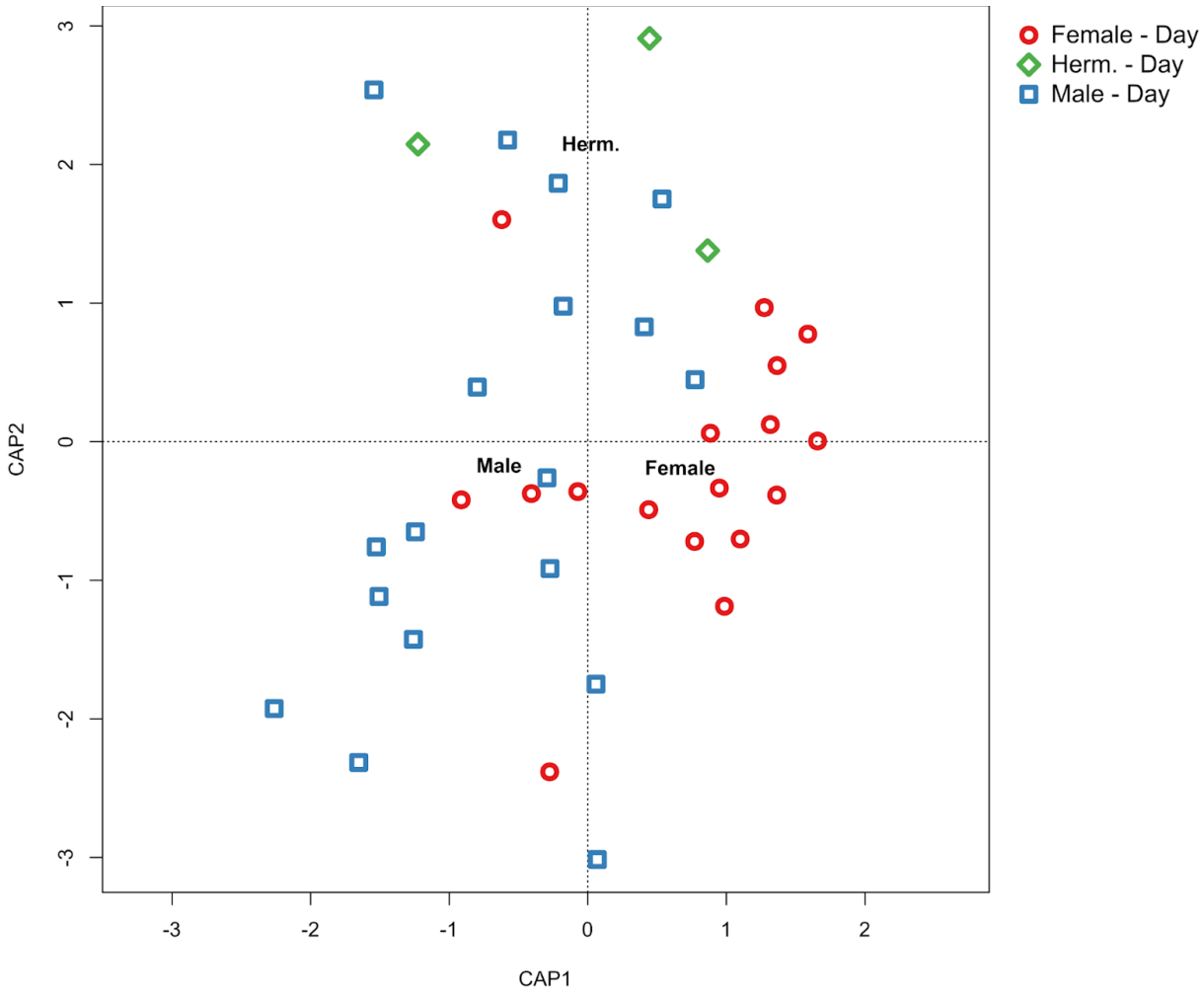
Canonical analysis of principal coordinates (CAP) of floral scent compositions (the emissions of each compound relative to total emissions) using Bray-Curtis distances and tested with 999 permutations. The analysis excludes hermaphrodites because of the low sample size. The predictors explained a total of 57% of the total variation (inertia).

Predictor	**df**	**SS**	**F**	**p**
Sex	1	0.388	2.67	0.008
Population	8	2.204	1.89	0.002
Sex * Population	4	0.677	1.16	0.244
Residual	21	3.5		

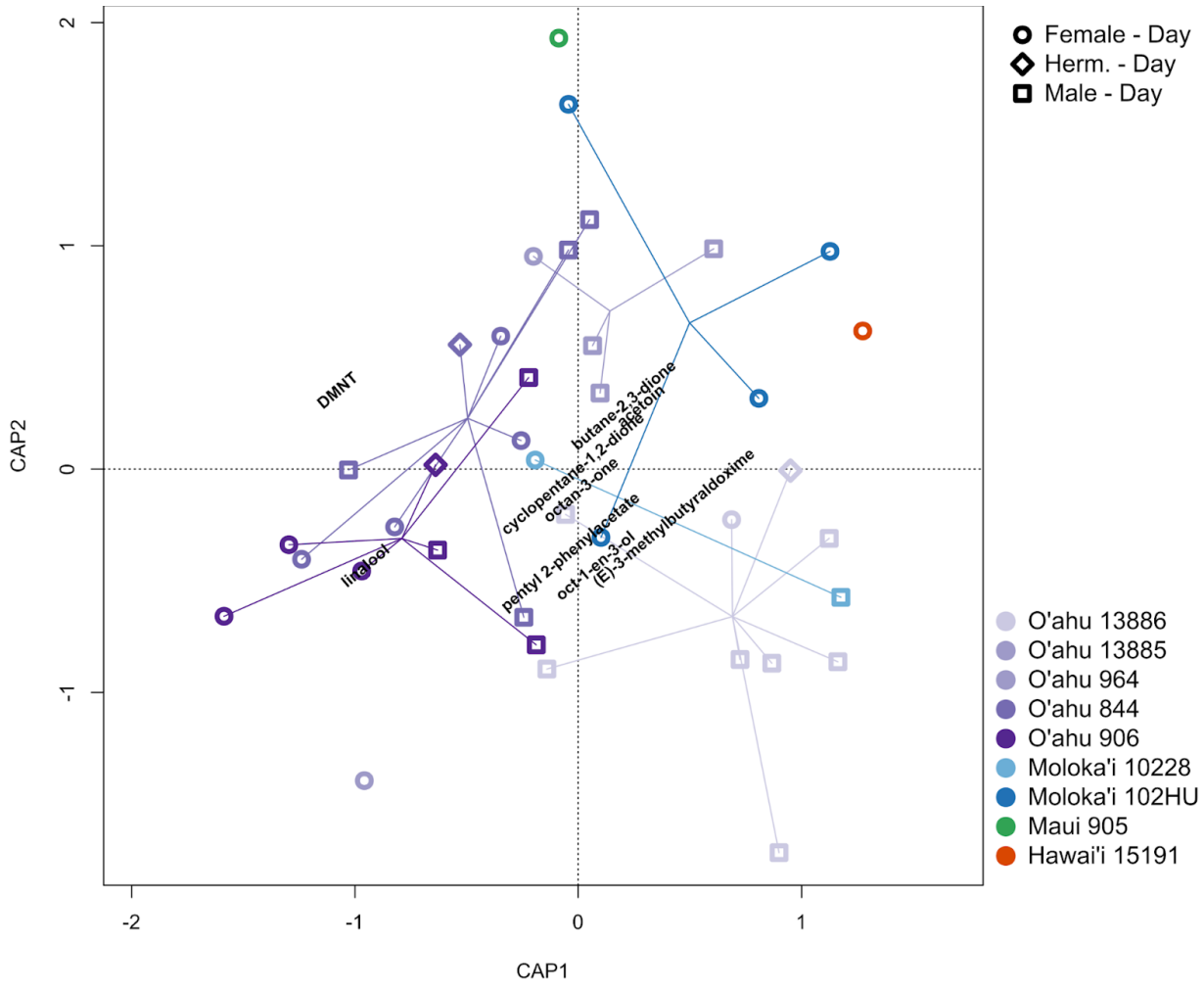
Ordinations of scent composition

CAP separating *Schiedea globosa* floral scent samples by sex (first panel) or population (second panel). The analysis used scent compositions (the emissions of each compound relative to total emissions) and Bray-Curtis distances. Sex (female, male, hermaphrodite) is indicated by shape. In the first panel the centroids of samples of the three sexes are labeled.

Scent samples separated by sex

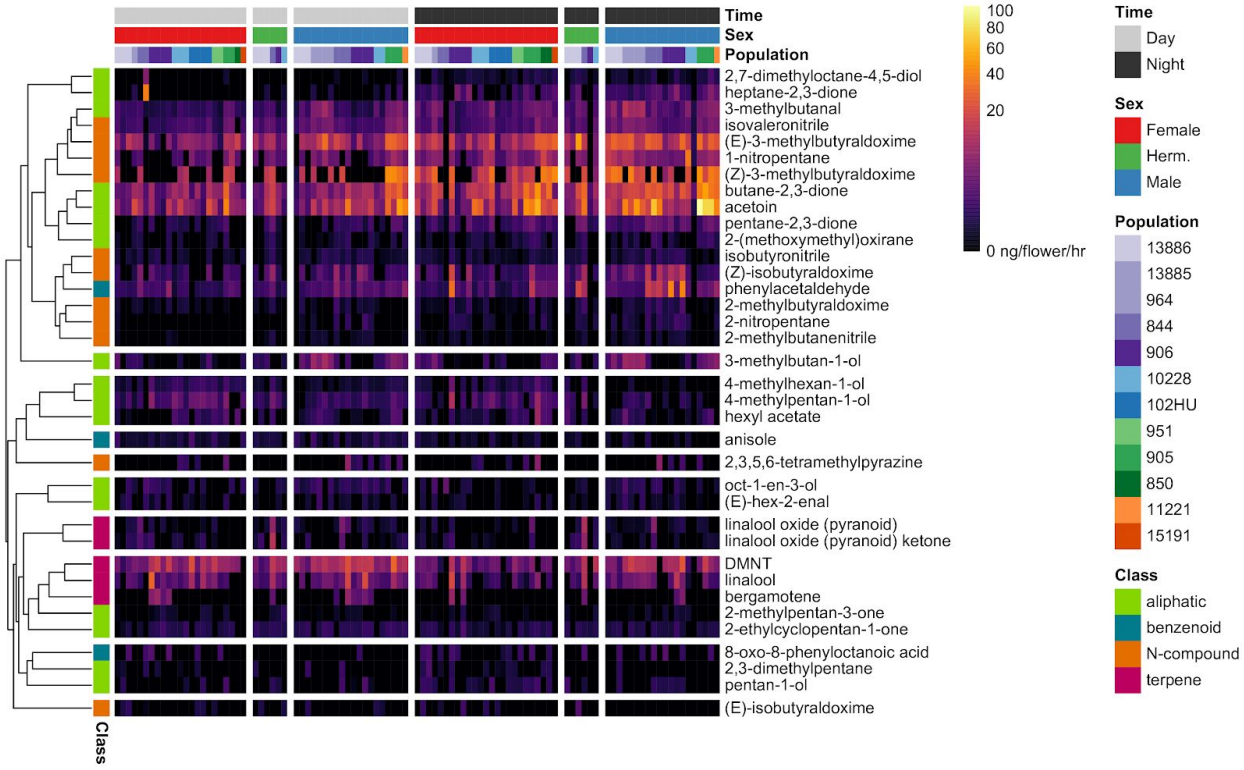


Scent samples separated by population



Appendix 3.S4: Heatmap of volatiles

Heatmap of *Schiedea globosa* volatiles that occur in at least 15 samples. Volatiles (rows) are clustered on the left by their emission patterns across samples by WPGMA hierarchical clustering of Pearson distances. The clustering is separated into 10 groups by cutting across the dendrogram at a uniform height. The compound class of each volatile is indicated by colors on the left bar. Samples (columns) are arranged by time, sex, and population, indicated by colors on the top bars. The heatmap shows emission rates per flower, represented by colors from black to yellow (low to high, scale on the right).



Appendix 3.S5: Genetic vs. geographic distance

Genetic distance versus geographic distance among populations. Mean intrapopulation genetic distances (for populations with more than one genetic sample) are shown as hollow circles. The grey, red, and black trendlines follow the pairwise distances among the following three sets of populations to examine the effect of long-range dispersal events (see Results and Appendix 3.S2): between the northern O‘ahu plant (Weller & Sakai 964) and all other populations (grey), between the two plants from Hawai‘i and all remaining populations (red), and among the remaining six O‘ahu and Maui Nui populations (black). A vertical line at 55 km divides the pairwise distances within and between historical islands, where Maui Nui (in this study, the current islands of Maui and Moloka‘i) is considered as one island. There was no statistically significant linear correlation between genetic and geographic distance overall (see Mantel tests in Results).