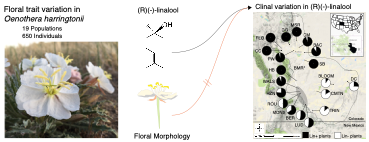
**Graphical Abstract**

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**Title**

Extensive population-level sampling reveals clinal variation in (*R*)-(-)-linalool produced by the flowers of an endemic evening primrose, *Oenothera harringtonii*

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**Abstract**

The study of floral trait diversity has a long history due to its role in angiosperm diversification. While many studies have focused on visual traits including morphology and color, few have included floral scent despite its importance in pollination. Of the studies that have included floral scent, sampling has been limited and rarely explores variation at the population level. We studied *Oenothera harringtonii,* which is primarily pollinated by hawkmoths and has flowers that open at dusk, produce nectar and are highly fragrant. We collected floral trait (morphology, scent chemistry and emission rates) data from 650 individuals from 19 wild populations and 49 individuals grown in a common environment. We identified 35 floral volatiles representing 5 biosynthetic classes. Population differentiation was stronger for floral scent chemistry than floral morphology. (*R*)-(-)-linalool was the most important floral trait differentiating populations, exhibiting a striking pattern of clinal variation across the distribution of *O. harringtonii* without any correlated shifts in floral morphology. Populations in the north and west produced (*R*)-(-)-linalool consistently, those in the east and south largely lacked it, and populations at the center of the distribution were polymorphic. Floral scent emissions in wild populations varied across four years but chemical composition was largely consistent over time. Similarly, emission rates in greenhouse-grown plants were lower but chemical composition was consistent with the wild population of origin. Our data set represents the most extensive population-level sampling of floral scent to date and suggests that such sampling may be needed to accurately capture the nature of variation in wild populations.

**Keywords**

*Oenothera harringtonii*; Onagraceae, Arkansas River Valley evening primrose, mass spectrometry; pollination; floral scent; floral morphology; monoterpenes; linalool

**1. Introduction**

The study of floral trait diversity has a long history due to its importance in understanding angiosperm diversification (Fenster et al., 2004; Ollerton et al., 2011). Variation in floral traits and the associated selective forces are considered a primary driver of speciation, commonly attributed to pollinator-mediated selection (Grant and Grant 1965, Stebbins 1974). While floral morphology and color have been extensively studied, comparatively little is known about the role that floral scent plays in mediating interactions with biotic agents of selection (Delle-Vedove et al., 2017; Raguso, 2008; Whitehead and Peakall, 2009). Including floral scent traits in studies of floral phenotypes is critical for developing a deeper understanding of floral evolution, especially when scent plays an integral role in pollinator attraction. The most detailed studies to date that provide evidence for scent-mediated diversification focus on extreme pollination specialization, including sexually deceptive pollination of orchids and brood-site mutualisms (reviewed in Raguso, 2004; Whitehead and Peakall, 2009), but much remains to be learned from more generalized systems (Larue et al., 2014) and those where visual and olfactory cues function in concert (Spaethe et al., 2007; Byers et al., 2014;). More recent work has revealed that floral scent is not a fixed, species-level trait; rather, it can be highly variable within a species, and can be explained by factors beyond pollinator-mediated selection (reviewed in Delle-Vedove et al., 2017).

Studies investigating variation in floral scent have tended to compare groups of related taxa to understand the role that scent may play in reproductive isolation and speciation (Byers et al., 2014; Chartier et al., 2011; Schiestl, 2015; Waelti et al., 2008). For example, work on the genus *Lithophragma* and their pollinating floral parasites, *Greya* moths, has revealed remarkable floral scent variation among clades, species, and populations (Friberg et al., 2019). In other systems, floral volatiles reflect taxonomic boundaries between otherwise morphologically similar taxa, including sexually deceptive *Chiloglottis* (Ebert et al., 2009) and *Orphys* orchids (Mant et al., 2005). Differences in floral scent among taxa have been shown to reduce the potential for introgression and to be an effective means of reproductive isolation between two cross-compatible species, *Silene latifolia* and *S. dioica* (Waelti et al., 2008). In other systems, hybrids have been shown to produce the same floral scents as parental taxa as well as transgressive compounds (those not produced by either parent; Chartier et al., 2016; Vereecken et al., 2010), providing opportunities for hybrids to attract new pollinators, enter unoccupied pollinator niches, and over time become reproductively isolated. While interspecific studies such as these provide insight into broad patterns of diversification, fewer studies provide robust assessments of variation at the intraspecific level, which are needed to fully understand the proximate drivers of diversification.

Populations commonly experience different biotic and abiotic environments in space and time, giving rise to geographic mosaics of selection that can produce intraspecific variation in traits (Thompson, 2005, 1999). Geographic patterns in intraspecific variation may signal diversifying selection and reflect areas with conflicting selective pressures for specific traits. Recent studies have focused on intraspecific variation to better understand the drivers of diversity at larger scales and evidence for divergent/differential selection. To date, intraspecific variation in floral volatiles has been attributed to selection by different pollinators (Anderson et al., 2010; Chapurlat et al., 2018; Gross et al., 2016; Gfrerer et al. 2021), differences in pollinator preference (Suinyuy et al., 2015), adaptation to heterogeneous pollinator communities (Szenteczki et al., 2021), and the impacts of larcenists or herbivores (Galen et al., 2011; Kessler et al., 2008; Theis, 2006), each with distinct and sometimes opposing impacts on plant fitness. Despite these advances, our understanding of intraspecific variation in floral scent remains limited. To date, most studies of floral scent include only a few (< 10) individuals per population and a small number of populations. Moreover, few of these studies investigate variation in space (sample the complete geographic distribution of the taxa of interest) and time (sampled over multiple years) (Delle-Vedove et al., 2017). Comprehensive sampling within populations and across space and time allows for a fuller understanding of the nature of variation, which then can inform predictions of underlying processes and mechanisms.

Here, we focus on a species with a limited global distribution, the flowers of which are visited by nocturnal moths that rely on scent to locate floral resources. *Oenothera harringtonii* (Onagraceae) W.L. Wagner, Stockh. & W.M. Klein is a self-incompatible annual that is endemic to the arid shortgrass prairies of south and central Colorado and adjacent New Mexico, USA (Fig. 1; Skogen et al. 2016). Thus, biotic pollination is crucial to its reproductive success, in a fragmented habitat that is vulnerable to urban sprawl and agricultural land conversion (Skogen et al. 2016). The primary pollinator of *O. harringtonii* across its natural range is the white lined sphinx moth, *Hyles lineata* (Sphingidae) (Skogen et al., 2016), an insect that is antennally sensitive to a diverse range of floral volatiles (Raguso et al. 1996), uses scent to locate flowers (Bischoff et al., 2015) and is also an herbivore whose larvae consume the flower buds and leaves of *O. harringtonii* (Balbuena et al. in press). Developing seeds are consumed by the larvae of another, more specialized moth species, *Mompha definitella* (Momphidae)*,* whose females oviposit on ovaries at dusk and are thought to use floral scent to locate *Oenothera* flowers, but do not pollinate them (see Artz et al. 2010). Pilot studies revealed striking geographic variation in floral scent composition of *O. harringtonii*, particularly in the presence or absence of linalool, a common volatile typical of hawkmoth-pollinated flowers, from which it is often emitted in large amounts (Raguso & Pichersky, 1999). We followed up with intensive sampling of floral traits across the full geographic distribution of *O. harringtonii,* seeking to better understand the patterns and processes related to floral scent polymorphism. From 2008-2012, we collected floral trait data (morphology, scent chemistry and emission rates) from 650 individuals in 19 natural populations (70% of all known occurrences; average of 30 individuals per population; see Table 1) and six populations grown under controlled greenhouse conditions. This high-density approach to floral sampling led to a robust characterization of variation in floral traits both within and among populations of *O. harringtonii*. Using these data, we explored 1) the geographic structure of variation in linalool and other floral traits and 2) the extent to which population-level variation in scent composition and emission rates is consistent (and thus potentially heritable) between field and greenhouse-grown plants from the same populations. . Our findings…(COME BACK TO THIS WHEN WE ARE DONE).

**2. Results and Discussion**

*2.1. Species-Wide Variation in Floral Traits*

A total of 35 floral volatiles were identified from 19 natural populations of *O. harringtonii* across the entirety of its natural distribution*.* These VOCs included 11 monoterpenoids, 11 sesquiterpenoids, 7 nitrogenous aldoximes and related compounds, 3 aromatics, two additional terpenoids (the diterpene-derived isophytol and (*E,E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene = TMTT) and the fatty acid derived “jasmine lactone” (7-decen-5-olide; Table S1). Most GC-MS traces were dominated by one or two monoterpenes, (*E*)-β-ocimene and (*R*)-(-)-linalool, combining for 80.7 to 97.2% of total ion chromatogram peak area across all populations. Previous analyses using chiral GC-MS had determined that linalool in *O. harringtonii* and related species is exclusively attributable to the (*R*)-(-)-linalool enantiomer (Bechen et al., in press) Occasional individuals from southeastern populations (MONS, WALS, DC, TRIN, CHAL/MAV) emitted large amounts of (*E,E*)-α-farnesene (Table S1). Remarkably, none of the remaining 32 VOCs accounted for more than 5% of total emissions in any population (Table S1). These minor constituents included terpenoids that were structurally similar to the 3 dominant compounds listed above (e.g. (*Z,E*)-α-farnesene, (*E*)-β-farnesene and (*E,E*)-farnesol), along with suites of biosynthetically linked metabolites, such as β-caryophyllene, α-humulene and caryophyllene oxide, and PHE-derived aromatics with (phenylacetonitrile, 1-nitro-2-phenylethane and phenylacetaldoxime) or without N-atoms (2-phenylethanol, phenylacetaldehyde)(Table S1). Nearly all these compounds are characteristic of night-blooming, hawkmoth-pollinated plants worldwide (Kaiser, 1993; Knudsen and Tollsten, 1993; Levin et al., 2003; Raguso et al., 2003), including other species of *Oenothera* (Raguso et al., 2007; Vergara et al. 2011). Two exceptions are methyl geranate and methyl farnesoate, representing terpenoid esters which are occasionally emitted by flowers but are better known from studies of insect sexual communication (Engel et al., 2016; see Schiestl and Ayasse, 2001 for farnesyl esters). The original descriptions of *O. harringtonii* as a discrete taxonomic entity emphasized its distinctive “gardenia-like” floral fragrance (Gocken, 1968; Wagner et al., 1985). Our analyses confirm that the combination of (*E*)-β-ocimene, linalool, methyl benzoate, (*E,E*)-α-farnesene, TMTT and jasmine lactone in floral headspace of *O. harringtonii* closely align with published scent profiles for *Gardenia jasminoides* (Cao et al., 2020).

*2.2. Population-Level Floral Variation*

When all floral traits were considered together, population differentiation was strongest for floral scent chemistry (ANOSIM: *R* = 0.40, p < 0.001) as compared with floral morphology (ANOSIM: *R* = 0.13, p < 0.001). A Random Forest analysis of floral morphology and scent compounds showed that one compound in particular, (*R*)-(-)-linalool, was the most important floral trait differentiating populations (Fig. 2). When present, (*R*)-(-)-linalool was also a dominant contributor to total volatile emission rates (Table 2, see 2.1), unlike other population-distinctive compounds such as jasmine lactone. For these reasons, we focused our further analyses and discussion on (*R*)-(-)-linalool, which is a common floral volatile in night-blooming, hawkmoth-pollinated plants (Kaiser, 1993; Knudsen and Tollsten, 1993; Raguso and Pichersky, 1999) and is a chiral compound (Raguso, 2016). Linalool has been identified in other night-blooming members of the genus *Oenothera,* including *O. acutissima* (sect. *Lavauxia*; (Raguso et al., 2007), *O. biennis* (sect. *Oenothera*; (Kawaano et al., 1995), *O. californica* (sect. *Anogra*), *O. cespitosa* (sect. *Pachylophus*), *O. howardii* (sect. *Megapterium*), *O. lavandulifolia* (sect. *Calylophus*) and *O. xylocarpa* (sect. *Contortae*); Jogesh, Skogen, and Raguso unpublished data). Flowers of *O. harringtonii* emit only the (*R*)(-) enantiomer (Bechen et al., In Review), and the available evidence suggests that floral emission of (*R*)-(-)-linalool is a plesiomorphic trait in *Oenothera* sect*. Pachylophus*, inherited by the ancestor of *O. harringtonii.* Hereafter, we refer to this compound as “linalool” for simplicity.

*2.3. Clinal Variation in (R)-(-)-linalool Emission*

Our data reveal a population-level linalool polymorphism, with a striking pattern of clinal variation across the geographic range of *O. harringtonii* without any correlated shifts in floral morphology. Emission of linalool was discrete and polymorphic; it was either completely absent /emitted in small quantities or constituted up to 95% of total floral emissions (Tables 1, 2). In northern and western populations, most plants (average of 86% per population) studied emitted linalool (lin+ populations) compared with only 22% on average in southern and eastern populations (lin- populations) (Fig. 1, Table 1). Emission rates varied between populations and years (pop: F18,628=3.90, p<0.01, year: F3,628= 71.62, p<0.01, Fig. 3A). Floral volatile composition was significantly different between lin+ and lin - populations (Nlin+ = 361, Nlin- = 289, ANOSIM *R* = 0.45, *P* = 0.001; Fig. 3B). Floral volatile composition was significantly different between years in CC and DC but not in PW and TRIN (CC ANOSIM *R* = 0.13, *P* = 0.001, PW ANOSIM *R* = 0.01, *P* = 0.35, TRIN ANOSIM *R* = 0.14, *P* = 0.03, DC ANOSIM *R* = 0.17, *P* = 0.001; Fig. 3C)

At the heart of this distribution are a few populations (HZN, MONS, ROU, BER, and LUD) in close geographic proximity, which most clearly represent the sharp northwest to southeast transition in floral scent chemotypes, as they included both lin+ and lin- plants in ratios that change abruptly over c. 75 km. Among the few individuals that produced linalool in lin- populations, emission rates were two orders of magnitude lower than those typical of lin+ populations (lin- populations have median linalool emission rates near zero, Table 1). Given that populations of *O. harringtonii* that produce linalool are in the western portion of its distribution, it is possible that linalool production may represent phylogenetic inertia (Raguso et al., 2006, 2003), as itis a local endemic species derived from a common ancestor of the widespread species *O. cespitosa* subsp. *marginata* (Patsis et al., 2021), whichoccurs across the western United States and is known to produce (*R*)-(-)-linalool (data not shown).

*2.4. Consistency Between Field vs Greenhouse-grown plants*

Other studies have documented plasticity in floral scent composition, emissions, and rhythms in response to abiotic factors such as temperature, light, humidity, soil, and drought (Friberg et al., 2014; Hansted et al., 1994; Jakobsen and Olsen, 1994; Majetic et al., 2007; Glenny et al. 2018; Campbell et al. 2019), prompting us to examine the potential for plasticity in *O. harringtonii* grown from seeds collected in 6 field populations (Table S2). Total emissions per flower per hour were not different between field and greenhouse-grown plants (pop: F5,274=3.89, p<0.01, origin field/ greenhouse: F1,274= 0.79, p=0.37, Fig. 4A), despite the presence of 10 fewer VOCs in the headspace of greenhouse-grown plants (Table S2). Floral scent phenotypes characterized from field-grown plants in lin+ populations were consistent in greenhouse grown plants from the same populations (Table S2; Fig. 4), suggesting that floral scent variation ~~in linalool production~~ has a genetic basis and is not plastic. Overall, floral volatile composition did not differ significantly between greenhouse-grown and field-collected lin+ populations (Nfield collected = 151, Ngreenhouse grown = 34, ANOSIM R = 0.05, p = 0.15) nor between greenhouse grown and field collected lin- populations (Nfield collected = 80, Ngreenhouse grown = 15, ANOSIM R = 0.07, p = 0.18; Fig. 4B). Nevertheless, the blend composition of minor scent constituents differed from that of field-collected samples in several ways. Greenhouse-grown plants produced four VOCs not identified from field-grown plants, including the (*Z*)- and (*E*)-furanoid linalool oxides, benzyl alcohol and methyl salicylate (Table S2). Conversely, some VOCs emitted at low-abundance by field-grown plants, including the (*Z*)- and (*E*)-pyranoid linalool oxides, all PHE-derived compounds (2-phenylethanol, phenylacetaldehyde, nitrogenous aromatics) and aliphatic aldoximes were absent in greenhouse-grown plants (Table S2).

*2.5. The Many Roles of Linalool*

*2.5.1. Linalool production and hawkmoth pollination*

Linalool is a common floral volatile in night-blooming, hawkmoth-pollinated plants (Kaiser, 1993; Knudsen and Tollsten, 1993; Raguso and Pichersky, 1999), including long-spurred orchids (Orchidaceae; Tollsten and Bergström, 1993), including Darwin’s orchid *Angreaecum sesquipedale* (Kaiser, 1993), members of the tomato family (Solanaceae; e.g. *Datura wrightii,* Riffell et al., 2009 *Nicotiana* spp., Loughrin et al., 1990), and jasmines (Oleaceae; *Jasminum* spp., Watanabe et al., 1993). Linalool has been shown to attract the widespread hawkmoth *Manduca sexta* in behavioral assays (Riffell et al., 2009), which uses *D. wrightii* as both a nectar source and larval host. Variation in linalool has been documented in other species pollinated by hawkmoths. For example, in South African *Gladiolus longicollis* (Iridaceae), positive directional selection on tube length results in populations with long or short morphs that are pollinated by long- and short-tongued hawkmoths, respectively (Anderson et al., 2010). Floral scent data collected in one population in a contact zone with both floral morphs revealed major differences in floral scent, whereby linalool was produced in plants with long floral tubes but not those with short floral tubes. Additional population-level sampling is needed to determine if floral scent differences are maintained beyond the contact zone and whether these differences contribute to different pollination ecotypes. Geographic variation also has been documented in floral VOCs derived from linalool. In moth pollinated *Linanthus dichotamus* (Chess et al., 2008) and *Abronia umbellata* (Doubleday et al., 2013) in California and *Silene latifolia* and *S. otites* (Dötterl et al., 2005a; Jhumur et al., 2007) in Europe, linalool is converted to lilac aldehydes and alcohols, which are known to attract noctuid moths but are less common than linalool in flowering plants (Dötterl et al., 2006). Linalool can function as a pollinator attractant or as a defense compound when emitted from floral tissues (Boachon et al., 2015; Okamoto, 2017; Raguso, 2016; Reisenman et al., 2010) and has been implicated in both direct and indirect plant defenses when emitted from vegetative tissues (Raguso, 2016), addressed in more detail below.

*2.5.2. Linalool and Floral Antagonists*

While many studies of floral trait variation have invoked pollinator-mediated selection, floral antagonists use some of the same traits to locate resources, with opposing impacts on fitness. The role of floral antagonists in generating floral trait diversity is relatively understudied but has been documented (Suchet et al. 2011, Galen et al. 2011) and it is possible that variation in scent in *O. harringtonii* may be the result of selection by antagonists that are attracted or deterred by specific compounds or bouquets. *O. harringtonii* is a known larval host for *H. lineata* (caterpillars consume flower buds and to a lesser extent leaves) and *Mompha definitella* (caterpillars are seed predators), both of which are likely to locate floral resources via fragrance. Female *H. lineata* have been observed to oviposit while nectaring and their larvae eat flower buds and sometimes leaves of *O. harringtonii*, as has been shown for *O. cespitosa* in western USA (Artz et al., 2010). Members of the Onagraceae are a known host for microlepidopteran moths in the genus *Mompha,* and *M. definitella* females oviposit in flowers and immature fruits and larvae consume developing seeds (Bruzzese et al., 2019). Both *H. lineata* and *M. definitella* are crepuscular and have been observed visiting flowers of *O. harringtonii* at dusk (data not shown).

*2.5.3. Factors influencing variation in linalool*

Intraspecific variation in floral scent has been attributed to many factors including differences in biotic agents of selection (pollinators and herbivores), mating systems, and genetic drift, among others (Delle-Vedove et al. 2017). In some systems, such as *Arum maculatum,* the maintenance of intraspecific variation in floral odors is thought to be advantageous when pollinator communities vary and do so over short time scales (Gfrerer et al., 2021; Szenteczki et al., 2021). However, geographic variation in floral scent in *O. harringtonii* appears unlikely to indicate pollination ecotypes or differences in pollinator climate (Grant and Grant, 1965; Van der Niet et al., 2014). Our previous studies revealed that *Hyles lineata* hawkmoths are the most consistent floral visitor and most effective pollinator and are reliable in both space (range wide) and time (multiple years) (Rhodes et al., 2017; Skogen et al., 2016), suggesting that the clinal variation in linalool is not driven by local adaptation by pollinators to this particular compound. Intraspecific variation in scent has been shown in other systems for which pollinators do not vary. Mant et al. (2005) observed significant inter-reginal differences in VOCs produced by flowers of the deceptive orchid *Ophrys exaltata* despite all studied populations being pollinated by *Colletes cunicularris* bees. They suggest that the pattern may be due to divergent selection resulting from differences in local preferences of pollinators*.* Similarly,Suinyuy et al (2012) documented clinal variation in volatiles emitted by cones of the cycad *Encephalartos villosus,* despite no difference in pollinator assemblages, a pattern that was initially attributed to genetic drift or coevolution. Subsequent work revealed that olfactory preferences of weevil pollinators matched cone volatiles in their local regions, consistent with expectations for brood-site mutualisms (Suinyuy et al. 2015). Lastly, variation in floral scent can play an important role in assortative mating (Dötterl et al., 2005b; Huber et al., 2005; Plepys et al., 2002), especially for plant species for which scent is the primary floral attractant, such as moths (Raguso, 2008; Waelti et al., 2008). While our greenhouse data suggest linalool is heritable, a formal heritability study and fine scale assessments of assortative mating in populations that are polymorphic for linalool would provide further eliminate the role of pollinator-mediated selection for linalool in this system.

It remains possible that linalool may influence the foraging behavior (through floral constancy or probing time) of *H. lineata* in a way that could contribute to the observed linalool polymorphism, if, for example, the lin+ populations of *O. harringtonii* experience greater competition from co-blooming plants to attract *H. lineata* as pollinators.

our prior genetic studies suggest that this is not the case. Hawkmoths use floral visual and olfactory cues to locate floral resources (Raguso and Willis, 2005), are known to travel over long distances (Alarcón et al., 2008; Finger et al., 2014; Stockhouse II, 1973), and therefore have the potential to make meaningful contributions to gene flow and limit differentiation via genetic drift. Our prior work in this system has documented extensive gene flow range-wide, low genetic differentiation and weak isolation by distance (Skogen et al., 2019). In addition, although there was a significant relationship between genetic and geographic distance (Skogen et al., 2019), we found that clinal variation in linalool was related to geographic distance (partial Mantel test: *R2* = 0.11, coefficient = 0.35, *t* = 4.54, *P* < 0.01) but not genetic distance (partial Mantel test: *R*2 = 0.11, coefficient = -2.49, *t* = -1.50, *P* = 0.30). These data suggest that the clinal variation in linalool documented here for *O. harringtonii* is unlikely to be explained by geographic variation in the foraging preferences of *H. lineata* for different floral scent bouquets, or genetic drift, suggesting that other biotic or abiotic factors are at play.

Last, linalool can be present in different chiral configurations and ecological contexts, is not restricted to hawkmoth-pollinated plants, and is not limited to a floral context (rev. by Raguso, 2016; Raguso and Pichersky, 1999). For example, (*S*)-(+)-linalool is a target of phenotypic selection, increasing seed fitness in bee-pollinated *Penstemon digitalis* (Parachnowitsch et al., 2012). Specific ratios of this enantiomer with other monoterpenes were shown to mediate obligate mutualism between *Ficus hispida* and its pollinating wasps (Chen and Song, 2008). Linalool is also a common vegetative volatile, either emitted constitutively by glandular trichomes or as a component of systemically released blends induced by herbivory (rev. by (Raguso, 2016). A transcriptome study of *O. harringtonii* revealed differential expression of linalool, primarily due to differences in genes expressed in petals and not leaves (Bechen et al., in press). However, the extent to which linalool is an attractant or deterrent to antagonists and whether it is constitutively expressed or induced in this system remain to be determined.

**3. Conclusions**

We provide the most comprehensive dataset of intraspecific variation in floral volatiles to date, which revealed clinal variation in linalool production in *O. harringtonii.* Such extensive sampling may be needed to accurately capture the nature of variation in wild populations, which has important implications for understanding floral trait evolution and for inferences of selective pressures and adaptation within and among species and at higher taxonomic levels. Now that floral scent is more commonly studied, it is clear that geographic variation in scent composition is a widespread phenomenon with many potential causes (rev. by (Delle-Vedove et al., 2017). When phenotypic variation is geographically structured, selection can lead to adaptive divergence. Future work is needed to assess the genetic basis of linalool variation in *O. harringtonii* and to explore the causal mechanisms that created the clinal pattern of linalool variation, with special emphasis on the relative contributions of the mutualistic and antagonistic roles of *Hyles lineata* and the antagonistic role of seed predation by *Mompha definitella* to phenotypic selection. More broadly, additional detailed studies of intraspecific variation in floral scent are needed to understand the extent to which this trait contributes to floral trait and angiosperm diversification and the roles of biotic and abiotic agents of selection.

**4. Experimental**

*4.1. Study System*

*Oenothera harringtonii* Wagner, Stockhouse, and Klein (Onagraceae) is endemic to the arid shortgrass prairies of south and south-central Colorado and adjacent New Mexico, USA. (Wagner et al., 1985)(Fig. 1). It is a facultative annual with white, fragrant flowers that produce copious nectar, open at dusk, and commonly last 14 -18 hours before fading the following morning. *O. harringtonii* is self-incompatible, relying exclusively on pollination for reproduction (Skogen et al. 2016). Gene flow is high and population differentiation is low range-wide (Skogen et al., 2019), likely facilitated by the primary pollinator, the white-lined sphinx moth, *Hyles lineata* Fabricius (Sphingidae, Skogen et al., 2016). Solitary bees also visit and pollinate flowers in the morning hours when most pollen has already been removed, and therefore make minimal contributions to fitness. Herbivores include larvae of *H. lineata*, who consume flower buds and to a lesser extent leaves, and *Mompha definitella* Zeller*,* who consume seeds and are host-specific on *O. harringtonii* (Bruzzese et al., 2019). Seeds are gravity dispersed likely explain patterns of local spatial genetic structure (Rhodes et al., 2014). *O. harringtonii* is a member of *Oenothera* section *Pachylophus* and is derived from the more widespread *O. cespitosa* (Patsis et al., In Press).

*4.2. Floral Phenotype*

We unbiased methods quantified chemical and morphological floral variation of *O. harringtonii* range-wide by sampling plants *in situ* in more than 70% of known populations over four years, 2009-2012 (650 individuals, 19 populations; Fig. 1 and Table 1). At each population, we collected floral scent and morphological data from one flower per plant from 9-30 plants, depending on the number of blooming plants found at each location. We sampled floral scent immediately after anthesis (between 18:00 and 20:00 hrs, when moth visits are most frequent) on individual flowers using dynamic headspace collection methods (Raguso and Pellmyr, 1998). Following scent collection, we excised the flowers from plants and measured floral morphology, then pressed flowers to (later) record dry masses.

*4.2.1. Floral scent*

Individual flowers were enclosed in a Reynolds (nylon resin) oven bag (12 x 15 cm, 270 ml volume) affixed to the floral tube or ovaries with plastic ties using protocols described by (Galen et al., 2011). Leaf material was never enclosed within headspace bags. Floral volatiles were collected in a cartridge containing 10 mg of Super Q (80–100 mesh) adsorbent (Alltech Associates, Waukeegan, Illinois, U.S.A.), packed into a Pasteur pipette using silanized quartz wool (Restek #24324). Ambient controls were collected each night that flowers were sampled; Reynolds oven bags enclosed a cartridge and were placed at least 3m from a plant with open flowers. Additional unused cartridges, carried to field sites in coolers, served as additional controls for any ambient contaminants associated with storage conditions. Air from the floral or ambient headspace, concentrated in the enclosing bag, was pulled through the cartridge using a PAS-500 (Spectrex, Redwood City, California, U.S.A.) personal air sampler vacuum pump, with a standardized flow rate of 200 ml air/min. After 60 minutes of sampling, the cartridges were removed and volatiles were eluted with 200 μl of GC-MS grade hexane (Honeywell Burdick and Jackson, Muskegon, Michigan, U.S.A) into borosilicate glass “shell style” inserts placed within 1.5 ml amber glass autosampler vials capped with silicone septa and polypropylene screw caps (Supelco, Bellefonte, Pennsylvania, U.S.A.). Samples were stored at -20 °C until analysis. Prior to analysis, we concentrated the samples to a uniform volume of 50 μl using gaseous N2 and added 5 μl of 0.03% toluene in hexane (= 23 ng) as an internal standard.

One µl aliquots of each sample were injected into a Shimadzu GC-17A gas chromatograph equipped with a Shimadzu QP5000 quadrupole, electron ionization (EI) mass spectrometer (Shimadzu Scientific Instruments, Columbia, Maryland, U.S.A.) as a detector. All analyses were made using splitless injections of 1 μL sample aliquots (1.5 min sampling time) onto a polar GC column (diameter 0.25 mm, length 30 m, film thickness 0.25 µm (Econo Cap's carbowax coating, known as EC WAX); Alltech Associates), using a mobile phase of ultra-high purity (99.999%) helium (constant flow of 1ml/min.). The GC program parameters (injection port temp. 240 °C, detector temp 260 °C, initial temp. 40 °C, hold time 2 min, increased at 15 °C/min to 260 °C, hold time 2.38 min) for a total run time of 19 min. per sample.

EI mass spectra (70 eV) were collected from m/z 40-350 (daltons) at a detector voltage of 70 eV, with scan speed of 1000 and a scan interval of 0.29 sec. GC peaks that were either absent in control samples or had at least 5-fold lower peak areas in controls were considered floral in origin. Compounds were tentatively identified using computerized mass spectral libraries (Wiley Registry of Mass Spectral Data, National Institute of Standards and Technology, and Adams (> 120,000 mass spectra)), and identifications were confirmed wherever possible through co-chromatography with authentic standards. Kovats retention indices (KI) were calculated for each identified VOC through comparison with the absolute retention times of n-alkanes, as outlined by Friberg et al. (2019). Calculated KI values were compared with compendia of published KI for each volatile compound using the NIST webbook (<https://webbook.nist.gov/>) and Pherobase (<https://www.pherobase.com/>; El-Sayed, 2021), narrowing the available KI values to those collected on a similar polar GC column using a linear temperature ramp (Tables S1, S2).

Peak areas were integrated using Shimadzu's GCMSsolutions software, were normalized for slight differences in final sample volume using the internal standard (see Galen et al. 2011) and were quantified by comparison with 5-point (log scale) dose-response external standard curves generated using serial dilutions (0.1 to 0.00001 mg/ml) of authentic standards for (*R*)-(-)-linalool. Emission rates were expressed as ng per flower or per gram dry floral mass (described below), per hour.

To assess whether floral chemotypes were true breeding, we grew plants of *O. harringtonii* from wild seeds collected from multiple maternal plants from each of six focal populations (FLO, Bloom, BAC, BMR, PW, and DC, Table 1) in 2008 and 2009 in the greenhouses at the Chicago Botanic Garden, Glencoe, Illinois, U.S.A. Seeds were sown in a 50:50 mix of field-collected soil and germination potting soil. Seedlings with true leaves were transplanted individually to 11.4 cm x 11.4 cm x 15.2 cm plastic pots. Plants were spaced evenly and randomly with respect to population of origin and grown with supplemental lighting to achieve 14 hr day/10 hr night (25oC day, 22oC night), and were fertilized weekly. Floral scent was sampled using the same methods described above from individuals from different maternal lines (unrelated individuals).

*4.2.2. Floral morphology*

Morphological measurements were made with digital calipers (nearest millimeter) and included corolla diameter (averaged along the two longest perpendicular axes), floral tube length (ovary to floral tube opening), floral flare (diameter of the floral tube opening), filament length and style length. We calculated herkogamy (anther-stigma separation) as the difference between the filament and style lengths. We then dried the flowers in silica and recorded dry weight to the nearest milligram. Dry weights were used to calculate emission rates/ng dried flower/hour (above).

*4.2.3. Statistical Analyses*

We took two approaches to understand how floral traits vary between wild populations. We first used a distance-based approach (ANOSIM) to understand if populations differed in their overall scent and morphological composition. Emission rates and morphology were square-root transformed and ANOSIM analysis was conducted using Bray-Curtis distances. We conducted separate ANOSIM analysis for floral scent and morphology. We then applied a Random Forest model to determine the traits most important in differentiating the populations from each other (Ranganathan and Borges, 2010). We tested for differences in scent composition ~~linalool production~~ between field and greenhouse-grown plants using ANOSIM analyses and compared emission rates with a general linear model with population and origin as predictors. Total emission rates log transformed to meet assumptions of normality. To determine if geographic patterns of linalool emission are driven by genetic differentiation (using pairwise genetic distances from (Skogen et al., 2019) ) while controlling for geographic distance, we conducted a partial Mantel test using the multi.mantel function implemented in R package ‘phytools’ (Revell, 2012). All analyses were conducted in R version 4.0.2 (“R: The R Project for Statistical Computing,” 2020).

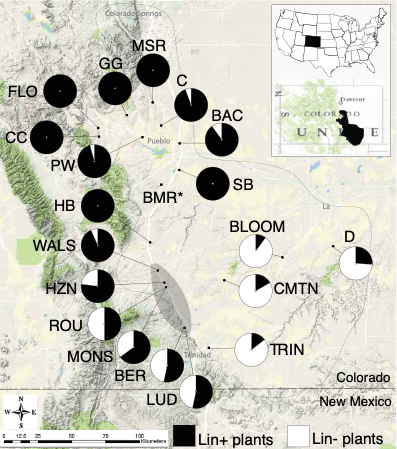
**Declaration of competing interest**

The authors declare no competing financial interest.

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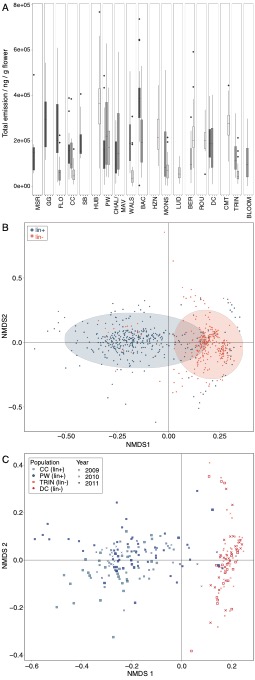
**Figures and Legends**

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**Figure 1.** Geographic distribution of the cline in linalool production across *O. harringtonii* populations. Pie chart­­­­­­s depict the proportion of individuals sampled in the field that emit linalool (lin+, black) and the proportion that do not (lin-, white) (Table 1). The gray oval indicates the portion of the distribution where plants are polymorphic for linalool production. Note that for the few linalool-emitting individuals in the lin- populations, emission rates are two orders of magnitude lower than those typical of lin+ populations (lin- populations have median linalool emission rates near 0, Table 1). Data for the BMR population were collected from greenhouse-grown plants only; population information and data can be found in Tables 1 and S2.

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**Figure 2.** Ranking of the relative importance of each trait based on the Random Forest classification algorithm for differentiating populations using data collected *in situ* (N = 650 plants). Gray circles represent morphological traits and black circles represent floral volatile traits. The importance (mean decrease in Gini coefficient) indicates the extent to which populations are not correctly differentiated if the trait is not included in the model. Thus, the higher the mean decrease in Gini, the more important the trait is for distinguishing populations.

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**Figure 3.** Floral scent data. (A) Boxplot of Total emission rates (ng/flower/hr + SEM) of volatile headspace from single flowers of *Oenothera harringtonii* collected in 19 populations across 4 years, (B) Non-metric multidimensional scaling plot (NMDS) of floral volatile composition. Each point represents an individual flower from one plant. Floral volatile composition was significantly different between lin+ and lin - populations (Nlin+ = 361, Nlin- = 289, stress = 0.14, R2 stress=0.90, ANOSIM *R* = 0.45, *P* = 0.001); (C) NMDS floral volatile composition of populations sampled in more than one year. Floral volatile composition was significantly different between years in CC and DC but not in PW and TRIN (CC ANOSIM *R* = 0.13, *P* = 0.001, PW ANOSIM *R* = 0.01, *P* = 0.35, TRIN ANOSIM *R* = 0.14, *P* = 0.03, DC ANOSIM *R* = 0.17, *P* = 0.001).

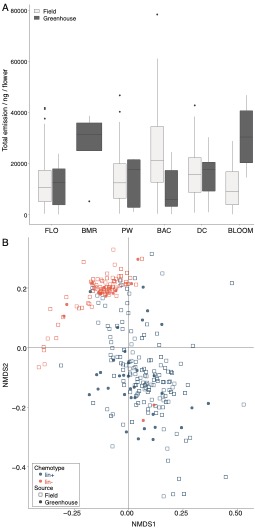
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Figure 4.(A) Total emission rates ng/flower/hour + SEM between field-collected and greenhouse-grown plants from four lin+ (FLO, BAC, PW, BMR) and two lin- populations (DC and Bloom), which nevertheless contained some plants producing low-levels of linalool (mean emission rates for DC were 100-fold (field) or 10-fold (greenhouse) lower, respectively than those of plants from FLO); NFLO, Field = 34, NFLO, Greenhouse = 15, NPW, Field = 68, NPW, Greenhouse = 5, NBAC, Field = 49, NBAC, Greenhouse = 9, NBMR, Greenhouse = 5, NDC, Field = 50, NDC, Greenhouse = 11, NBLOOM, Field = 30, NBLOOM, Greenhouse = 4. Note that emission rates are per flower per hour and not per gram of dry flower as in Fig. 3A, because dry floral masses were not collected for the greenhouse plants. (B) Non-metric multidimensional scaling plot (NMDS) of volatile headspace from single flowers of *Oenothera harringtonii*. Floral volatile composition was not significantly different between greenhouse grown and field collected lin+ populations (Nfield collected = 151, Ngreenhouse grown = 34, ANOSIM *R* = 0.05, *P* = 0.15) nor between greenhouse grown and field collected lin- populations (Nfield collected = 80, Ngreenhouse grown = 15, ANOSIM *R* = 0.07, *P* = 0.18). Data are square root transformed chromatographic peak areas expressed as relative percentages of total emissions. The NMDS plot compares chemical space loci for volatile blends from the flowers of greenhouse grown (filled symbols) plants with seeds collected from lin+ (blue: FLO, BAC, PW, BMR) and lin- (red: DC and Bloom) populations to emissions from flowers sampled in the field from the same six populations, except for BMR (data collected in the greenhouse only).

**Table 1.** Population name, code, chemotype and location for all populations included in this study. Sample sizes are provided for the following data collected *in situ:* floral traits (floral scent and morphology data), for the years data were collected. \*Floral scent for the population BMR were collected from plants grown under common greenhouse conditions and were not collected *in situ*. Populations are arranged by proportion of individuals emitting linalool (highest at top) and then by latitude for populations where linalool was produced in all individuals.

Table 2: Floral scent collected in situ from wild populations of *Oenothera harringtonii*, during the first hour of anthesis (single flowers). Compounds shown in bold font were confirmed through co-injection with authentic standards. Other compound identities are inferred from mass spectral library match and co-occurrence with related compounds or isomers. Enantiomeric configuration of linalool was determined in a previous study for multiple populations using chiral GC-MS (Bechen et al. in press). Data shown are mean +/- std. error (s.e.m.) % of total emissions (TIC peak areas) for each compound. Population abbreviations and order (proportion of plants emitting linalool) follow those presented in Table 1.

1. m/z 41(100), 67(73), 83(45), 43(43), 69(35), 55(33), 82(25), 53(21), 54(14), 81(14)

2. m/z 79(100), 81(37), 41(28), 77(26), 43(24), 39(19), 53(13), 72(11), 93(11), 91(9)

3. TMTT = 4,8,12-trimethyl-1,3,7,11-tridecatetraene; stereo configuration not determined without an authentic standard

4. Jasmine lactone = 7-decen-5-olide

Table 3: Floral scent collected during the first hour of anthesis from single flowers of *Oenothera harringtonii* plants grown in the greenhouse.

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**Data, code, and materials**

Original data and code created for the study will be available in a persistent repository upon publication.