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Research article

Generalist-pollinated *Arabis alpina* exhibits floral scent variation at multiple scales

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Plants that depend on animals for reproduction often use complex floral traits to attract pollinators. Floral scent is recognized as part of the pollinator attraction module and can be shaped by plant-pollinator interactions. In recent decades, research has started to reveal the dynamic properties of floral scent, identifying patterns of spatial and temporal variation in floral scent emissions at various scales. Here, we investigate the levels at which floral scent varies in two populations of the generalist, perennial herb *Arabis alpina* (Brassicaceae) and if scent variation co-varies with pollinator activity, which would be expected if scent production is costly. First, we show the potential for both floral scent and pollinator communities to vary at a small geographic scale, between neighboring populations located some 4 km apart. Then, we investigate diel variation in floral scent emission rate and pollinator activity to test for synchronization between plants and pollinators. Further, we sampled volatiles from dissected floral parts to determine where floral scent compounds are produced in *A. alpina*. The two populations were pollinated by partly different communities of diurnally active insects, and scent composition, specifically petal and reproductive organ scent, differed between the two neighboring populations. However, we found no evidence of a diel synchronization between floral scent emission and insect activity, as *A. alpina* emits similar amounts of scent regardless of time of day and temperature. Whereas the spatial variation (within flowers, among populations) suggests specific and localized functions of the floral scent, the constant and stable scent emission indicates a low production and maintenance cost of the floral volatiles in this system.

Keywords: *Arabis alpina*, diel floral scent rhythms, floral scent variation, intraspecific variation, pollinator community

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Introduction

Plants have evolved floral traits that attract pollinators (Stebbins 1970, Caruso et al. 2019), including color patterns and other morphological structures (e.g. shape, size) (Harder and Johnson 2009), as well as olfactory cues (floral scent) (Raguso 2008a, Farré-Armengol et al. 2015). The role of floral scent as a multidimensional mediator of plant insect interactions has received increased attention during the last couple of decades (Raguso 2008b, Schiestl 2015). A growing body of evidence suggests that floral scent is of particular importance in many specialized pollination systems (Andersson et al. 2002, Salzmann et al. 2006, Chen et al. 2009, Svensson et al. 2011, Friberg et al. 2014). In addition, generalized plants that interact with multiple functional pollinator groups also use floral scent signals to advertise rewards (Theis 2006, Raguso 2008a, Johnson and Hobbhahn 2010, Larue et al. 2016), and potentially to guide pollinator behavior during the interaction (García et al. 2021).

One emerging pattern from studies of the ecology and evolution of floral scent is how chemical cues emitted by flowers can vary along various spatial and temporal scales. On spatial scales, several recent studies report ample variation in the strength and composition of the floral scent signals among populations of the same plant species (Majetic et al. 2008, Sun et al. 2014, Gross et al. 2016, Friberg et al. 2019, Gfrerer et al. 2021, Petrén et al. 2021), which is often hypothesized to reflect local specialization to spatial variation in pollinator communities (Wright and Schiestl 2009, Gross et al. 2016). Although inter- and intraspecific floral scent variation may result from neutral processes such as genetic drift (Friberg et al. 2019, Powers et al. 2022), or can be subject to non-pollinator mediated selection (Knauer and Schiestl 2017), a plethora of studies demonstrate connections between floral scent and pollinator activity. Specifically, across many systems, floral scent signals mediate and manipulate pollinator behavior (Svensson et al. 2011b, Rachersberger et al. 2019). Although not all compounds present in the floral scent emission are bioactive (Raguso 2009), pollinators are often highly responsive to particular compounds (Dodson et al. 1969, Andersson and Dobson 2003, Hoballah et al. 2005, Wright and Schiestl 2009, Byers et al. 2014). As such, selection on floral scent compounds has been studied in a handful of natural systems (Parachnowitsch et al. 2012, 2013 Gross et al. 2016, Opedal et al. 2022) in experimental trials (Gervasi and Schiestl 2017), and in one case experimentally attributed to pollinators as selection agents (Chapurlat et al. 2019). While these studies highlight the importance of floral scent in mediating plant-insect interactions, we are still only beginning to understand how insects shape the observed floral scent variation, and the scales at which we can detect variation in floral scent among populations and individuals.

Floral scent emissions can vary in time at different scales. Across days or season, scent may vary as a function of plant ontogeny or due to active or passive responses to environmental conditions such as temperature shifts (Goodrich et al. 2006, Friberg et al. 2013, Delle-Vedove et al. 2017). Within the course of a day, many species vary in the quantity or composition of scent emitted. Diurnal synchronization between floral scent emission and pollinator activity may have evolved in response to ecological costs of floral scent emission, by maximizing visits of beneficial floral visitors while minimizing detectability of antagonistic insects (Theis et al. 2007). Support for this hypothesis comes from studies reporting a match between periods of high scent emission and pollinator activity – which can be determined from either traditional pollinator observations or through temporal pollinator exclusion – (Raguso et al. 2003, Raguso 2004, Hoballah et al. 2005b, Theis et al. 2007, Friberg et al. 2014, Chapurlat et al. 2018, Burgin et al. 2023), although other species show very little diurnal variation in floral scent signals (Theis et al. 2007, Waelti et al. 2008). Some species regulate the total scent emission by emitting target-specific compounds, i.e., compounds recognized by the most efficient and/or abundant pollinator(s) that match the daily activity patterns of different pollinators (Hoballah et al. 2005, Chen et al. 2009). Another potential source of selective pressure on diurnal scent emission is the metabolic cost of scent production. Thus far, most examples available include plant species that are pollinated both during the day and at night (Chapurlat et al. 2018, Powers et al. 2020, Joffard et al. 2022), but if floral scent emission is metabolically costly, then exclusively daytime or nighttime-pollinated species would be predicted to reduce floral scent during diel periods of low pollinator activity. To date, the few studies that have attempted to identify metabolic costs involved in scent production have not been able to show strong costs of scent emission (Friberg et al. 2017, Luizzi et al. 2021).

At even finer scales, qualitative and quantitative variation in compound emissions can be found among floral tissues (e.g. petals, sepals and reproductive organs) (Pichersky et al. 1994, Piechulla et al. 2006, Friberg et al. 2013, Burdon et al. 2015, García et al. 2021). This pattern has been suggested to function similar to short-distance visual nectar guides, manipulating visiting insects to make contact with the reproductive floral structures (García et al. 2021). For many species, visual structures (such as petals) emit more of known pollinator attracting aromatic compounds such as benzenoids, phenylpropanoids and/or N-bearing compounds (García et al. 2021). Together, this suggests that plants can regulate scent emissions on small temporal scales while also compartmentalizing the location(s) of emissions, allowing for efficient signaling to pollinators on relevant scales.

Collectively, ecological and evolutionary studies of floral scent indicate that spatial and temporal changes in floral scent emission appear to be species-specific and/or context dependent. Here, we study the emerging model species, *Arabis alpina* (Brassicaceae) (Wötzel et al. 2022), where common garden studies show that population-level floral scent variation is primarily genetically determined (Petrén et al. 2021), and where the floral scent signal is highly canalized in response to environmental variation (Luizzi et al. 2021). In this species, the total emission rate and the composition of floral scent vary at multiple scales: among populations, and across mating systems (Petrén et al. 2021). These patterns suggest that floral scent variation is influenced by the extent of dependence on pollinators for reproduction. The white flowers are radially symmetric with a cruciform corolla, making them accessible to a wide range of pollinators. In fact, unpublished data identify insects from several orders visiting *A. alpina* populations in Italy and Greece (Petrén 2020, Thosteman 2024), suggesting a system where several different pollinator types contribute to pollination. To date, no formal studies have documented the major visitors of *A. alpina* flowers, and to what extent these vary among populations or in diel activity patterns. Similarly, we do not know to what extent floral scent variation among populations is explained by a uniform shift across floral tissues, or whether population-level variation at this small scale is primarily explained by the divergence of scent emission from only some tissues (c.f. Friberg et al. 2013, García et al. 2021). Investigating diel patterns of scent emission and composition in a generalist herb where these traits are known to vary among populations may inform the field of chemical pollination biology on the scales of local evolution in floral scent signaling.

To investigate diel patterns of floral scent emission and if these are synchronized with pollinator activity patterns, controlling for effects of temperature, we combine greenhouse floral scent experiments and natural pollinator exclusion experiments. We compare two pollinator-dependent, self-incompatible Italian populations which are located at different elevations, but only 4.2 km apart, in a region where floral scent is known to vary (Petrén et al. 2021). We ask 1) at what spatial and temporal scales we can detect variation in floral scent in the two neighboring populations of *A. alpina*, 2) to what extent there is detectable variation also in the local pollinator communities, and 3) how diel variation in pollinator activity corresponds to variation in floral scent, based on pollinator exclusion experiments. Finally, 4) we dissect the floral scent variation further by asking if particular floral tissues are responsible for population-divergence in chemical signaling.

Material and methods

Study plant and populations

Alpine rock-cress (*Arabis alpina* Brassicaceae) is a perennial herb occupying wet and gravel-rich arctic-alpine habitats across the Northern Hemisphere (Koch et al. 2006, Mossberg and Stenberg 2010). It grows in clusters of basal rosettes that form several 10–20 cm tall inflorescences with sparse clusters of white flowers (Fig. 1b, Mossberg and Stenberg 2010). In this emerging model system (Wötzel et al. 2022), previous research has explored the considerable intraspecific variation in floral scent (Petrén et al. 2021), mating system (ranging from self-compatible and selfing populations to self-incompatible and outcrossing populations) (Ansell et al. 2008, Tedder et al. 2011, 2015, Laenen et al. 2018) and floral morphology (Tedder et al. 2015, Toräng et al. 2017, Petrén et al. 2021). Typically, self-compatible *A. alpina* populations are distributed across mountainous regions in Northern Spain, the French and Swiss Alps, and the Norwegian and Swedish Scandes (Tedder et al. 2015, Toräng et al. 2017, Laenen et al. 2018). These populations generally have smaller flowers and emit less floral scent in comparison to self-incompatible populations in Italy and Greece (Petrén et al. 2021). In the Apennine mountains of the Abruzzo region, Italy (Fig. 1a) floral scent composition is known to vary at small spatial scales (~10 km) (Petrén et al. 2021). Italian populations are predominantly outcrossing, but minimal leakage in the SI-system can be observed. Seeds produced through selfing in these individuals are typically of low viability (Petrén et al. 2023).

We chose two focal *A. alpina* sites, located in the Apennine mountains only 4.2 km apart with an altitudinal difference of 800 m a.s.l. One of these sites, referred to as It4, is located 900 m a.s.l. (42°15′7.93″N, 13°19′8.54″E), and was included in the floral scent study by Petrén et al (2021). The neighboring site, It10 (42°14′18.20″N, 13°21′58.09″) was chosen based on it being geographically close but differing in habitat. The lower-altitude, It4 population is located in a valley at 900 m a.s.l., partly shaded by deciduous trees along a gravel road and surrounded by small scale farmland. In It4, *A. alpina* plants grow vertically on a natural rock wall along the road, as well as along the gravel roadside. The higher-altitude population, It10, is located 1700 m a.s.l. growing partly in less disturbed open grassland habitat and partly in a sparse beech forest. Here, plants grow in patches in grassy areas and between small rocks and boulders.

Pollinator exclusion experiment

In the spring of 2022, before the onset of flowering, we haphazardly selected plant individuals across the distribution of each focal population and used a fine mesh net to exclude pollinators by wrapping the net around the flower stem. We divided plants into four different treatments: negative control (always enclosed), day open (pollinators allowed between 08:00–20:00 h), night open (pollinators allowed between 20:00 and 08:00 h) and positive control (always accessible to pollinators, no netting). Treatment plants were always chosen in pairs growing no more than 50 cm apart, occasionally accompanied by a third plant used as positive or negative control. The pollinator exclusion experiment proceeded throughout the flowering period of the selected individuals (18 April–4 June 2022), and at the end of the season any buds or open flowers left on the plants were removed. We returned to the field sites approximately six weeks after the end of the flowering season and collected the fruits. The final number of individuals per treatment in It4 were 18 (day open), 12 (night open), 19 (positive control), 3 (negative control) and in It10 25 (day open), 22 (night open), 27 (positive control), 3 (negative control). We counted seeds from each fruit individually and report the results in mean number of seeds per fruit at the level of plant individual.

Insect visitor community quantification

We surveyed the insect communities in It4 and It10 during peak insect activity between 08:00 and 16:00 h, across the entire flowering period in 2022. Here, we use the term ‘insect

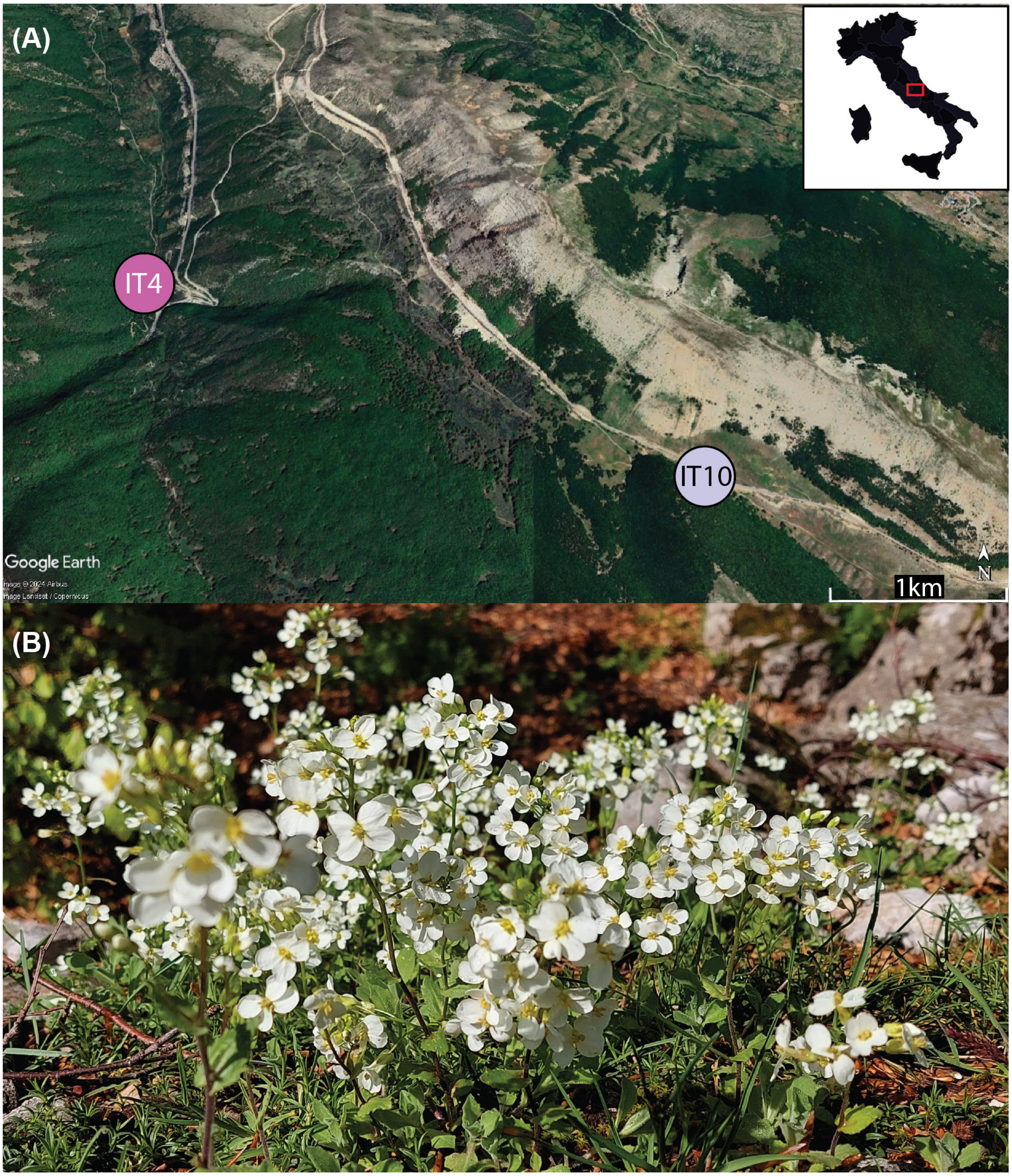

Figure 1. Focal *Arabis alpina* populations. (a) Location of the two populations used in the experiment. The geographic distance between the two populations is 4.2 km. Map based on Google Earth images. (b) A patch of *A. alpina* growing in It10. Photo: Hanna Thosteman.

visitor' as not all floral visitors contribute a pollination service (Stebbins 1970, Gómez et al. 2022). We repeated surveys at different hours of the day and at different locations within the population, which resulted in a total of 19 observation hours (It4: 9 h, It10: 10 h) evenly distributed across the entire flowering season. In every 1-h survey, a random patch of flowers was observed, the number of flowers counted, and all visiting insects recorded and identified to the lowest possible taxonomic level either on site or caught outside of survey hours, so as not to bias observations during the survey period, to be identified later. On three occasions (once in It4 and twice in It10), two 1-h surveys were conducted on the same day but on different flower patches. Taxon specific visitation rate was calculated as visits/flower/hour. We conducted no systematic nocturnal observations as *A. alpina* flowers tend to slightly close their petals at nightfall (H. Thosteman, pers. obs.). However, during one instance of nocturnal pollinator observation as well as on all visits to these or other *A. alpina* populations at dusk/night we observed no noctuid pollination events, or any pollinator activity.

Greenhouse common garden

Experiments took place in 2022 (It10) and 2023 (It4). Field collected seeds from two populations (5–9 seed families per population and 5 seeds per seed family) were sown in wells onto a soil mixture containing two thirds potting soil ('YrkesPlantjord' Weibulls Horto AB, Sweden) and one third 2–6 mm porous clay pearls (LECA, Saint-Gobain Byggprodukter AB, Sweden), with a top layer of moist sowing soil ('Plugg och Såjord', Weibulls Horto AB, Sweden). Planted pots were moved into a dark stratification chamber maintaining 4–6°C for 7–10 days before being transferred to a climate-controlled cabinet (Microclima-Series Economic Lux Chamber, Snijders Labs, Netherlands) maintaining 20°C 16-h days (120 µmol m^{-2} s^{-1}) and 16°C 8-h nights. Emerging seedlings were transplanted into individual pots containing the soil mixture described above. Seedlings were then allowed foliar growth for 6 weeks before being transferred into a room maintaining 8-h, 25–30 µmol m^{-2} s^{-1} 7°C days, and 16-h 5°C nights for vernalization. After 13–16 (It10) or 44–45 weeks (It4) the individuals were moved back into the controlled climate chamber which was programmed to maintain a light regime mimicking dawn, day, dusk and night (climate cabinet program: Night, 10 h darkness, 16°C; Dawn, 0.5 h half-light, temperature increase from 16–18°C; Day, 13 h full light, 18°C; Dusk, 0.5 h half-light, temperature decrease from 18–16°C). *Arabis alpina* plants typically require between 12–16 weeks of vernalization (Tedder et al. 2011, Toräng et al. 2017, Petrén et al. 2021b). The longer vernalization period for It4 was caused by logistic constraints, and previous greenhouse common garden experiments show no difference in plant development caused by longer vernalization (H. Thosteman, K. Eisen, M. Friberg, pers. obs.). Plants were watered regularly and nutrients (3‰ SW Horto 'SW Bouyant' RIKA S 7-1-5+Mikro) were added once a week throughout the flowering period. Due to issues with growth and flowering conditions, It4 plants were re-potted into 3 cm wider and 3 cm taller pots to enhance flowering, caused by the longer vernalization period.

Diel patterns of scent emission

Scent sampling from It4 and It10 plants followed an identical protocol and was performed under standard greenhouse conditions to eliminate effects of plasticity. We sampled 16 individuals representing 9 seed families from It10, and 17 individuals representing 5 seed families from It4. Scent was captured for three consecutive hours at dawn (light regime from dark to light including temperature increase), day (full light regime), dusk (light regime from light to dark including temperature decrease) and night (complete darkness) from the same individuals over a 24-h period. Thus, plants were given a minimum of 2 h to recover between sampling efforts.

Volatile collection was achieved by enclosing an inflorescence with known number of open flowers in a thin plastic bag (ICA AB 'Stekpåsar' and Toppits® 'Stekpåsar 2 in 1') and inserting a narrow Teflon tube containing a scent trap of 10 mg Tenax GR filter into the bag. The scent trap was connected to a plastic tube leading to a flowmeter (Cole-Parmer 65 mm direct-reading, Vernon Hills, IL, USA) and then to a Micro Air Sampler pump (Spectrex, Inc., Redwood City, CA, USA). Air was drawn from the bag and through the trap at a constant rate of 200 ml/min for three consecutive hours. For every scent collection bout, we added an ambient air control (empty bag) that was handled in the same way as the scent samples. Volatile sampling was performed in a separate chamber, with the identical light regime as the growth chamber, to avoid capturing volatiles from flowering non-target plants.

After collection, floral volatiles were eluted from the trap in 300 µl of hexane (GC grade 99% purity) and stored at −20°C before analysis. In preparation for Gas Chromatography/Mass Spectrometry (GC/MS), samples were concentrated to 50 µl by gently evaporating hexane by a moderate flow of nitrogen gas. Finally, 5 µl of 0.03% toluene was added to each sample as internal standard. Samples were analyzed in splitless mode using GC/MS on an Agilent 8890 gas chromatograph equipped with a 30 m × 0.250 mm × 0.25 µm HP-INNOWAX column (Agilent Technologies, Santa Clara, CA, USA) with ultra-high purity (99.999%) helium as a carrier gas at a constant velocity of 1 ml min^{-1}. The GC was linked to an Agilent 5977B mass spectrometer (Agilent Technologies, Santa Clara, CA, USA). An injection temperature of 260°C initiated the program, where column temperature was initially maintained at 50°C for 3 min before increasing to 250°C at a rate of 10°C min^{-1} for 20 min, where it was then held constant for 7 min until the end of the program.

The resulting chromatograms were manually integrated using ChemStation (ver. D 00.01.27, Agilent Technologies, Santa Clara, CA, USA) and peaks were identified using library suggestions (NIST MS Search 2.0, 2008). Compounds were verified using Kovats retention indices obtained from literature and occasionally by synthetic standards (Supporting information). In 2021, Petrén et al. identified 32 floral scent compounds present across 17 populations of *A. alpina*. Visual inspection of our chromatograms confirmed this, and we therefore focused on the 32 compounds in our analyses. Compounds with emission rates lower than three times the corresponding control sample (ambient air) were excluded from further analysis (Eisen et al. 2022b). Finally, all emission rates were converted to nanograms per hour per flower (Svensson et al. 2005). After filtering, 62 samples from It10 representing 16 individuals, and 64 samples from It4 representing 17 individuals were included in the statistical analyses.

Effects of temperature on floral scent emission

Floral scent emissions may vary with light, temperature and their interaction (Nielsen et al. 1995, Friberg et al. 2014, Jürgens et al. 2014). Volatiles of different molecular origin may also respond differently to changes in the abiotic environment, with some showing strong cyclic patterns and others being emitted at constant rates irrespective of, for example, temperature (Nielsen et al. 1995, Friberg et al. 2014). Thus, the aim of this experiment was not to replicate the natural conditions of the *A. alpina* populations, but to test the direct effects of temperature and light on the floral scent emission separately. To control for potential changes in scent emission caused by changes in temperature, we collected scent from four It4 *A. alpina* individuals placed in a chamber maintaining 5°C at both day and night, a temperature more similar to the night conditions experienced by the plants in their natural environment in April and May when they flower (Climate-Data.org 2024). We determined a sample size of four individuals to be sufficient to detect strong effects of temperature on floral scent emission rate (c.f. Steiner et al. 2011), under conditions which would allow no pollinator activities in natural *A. alpina* habitats. Scent was collected either in full light (25–30 μmol m^{-2} s^{-1}) (day) or complete darkness (night). Plants were given a minimum of 2 h to adjust and recover between scent collections and shifts in light regime.

Tissue specific scent emission

To identify spatial separation in compound emissions among floral tissues, we extracted scent from dissected flowers using Solid Phase Micro Extraction (SPME). Here, we sampled five individuals representing five seed families from It10, and nine individuals representing three seed families from It4. Two flowers from each individual were picked for sampling, where one was kept intact (whole flower, WF) and the other was dissected into petals (PE), sepals (SE) and reproductive organs (nectaries, stamens and pistil, RP). The whole or dissected parts of the flower were put separately in clean 2.5 ml glass vials and the opening was covered with one layer of thin plastic (ICA AB 'Stekpåsar' and Toppits® 'Stekpåsar 2 in 1'). Samples were left to equilibrate for 60 minutes before a syringe equipped with a super absorbent filament (100-µm polydimethylsiloxane fiber, SUPELCO Portable Field Sampler, Sigma Aldrich) was inserted into the vial for a 30 min exposure, after which the filament was removed from the vial, secured, and transported to the GC/MS for extraction. Occasionally, the syringes were stored in a fridge wrapped in plastic (see above) at 6°C for a maximum of 48 h before analysis. For every collection bout, we collected ambient air from an empty glass vial handled in the same way as the samples. The GC/MS protocol, and identification and quantification of chromatogram peaks were identical as for samples collected using dynamic headspace except from injection temperature which was 250°C for SPME samples. The results provided information on relative compound abundance in each sample.

Statistical analyses

All statistical analyses were performed using R (ver. 4.3.1) and R Studio (ver. 2023.06.2) (www.r-project.org).

Pollinator exclusion experiment

To test if the mean number of seeds per fruit differed between treatments, we performed a Kruskal-Wallis test followed by Dunn's test with Bonferroni correction for multiple tests, as the response variable (mean number of seeds per fruit per individual) did not meet the assumption of normality.

Insect community quantification

When quantifying variation among insect communities, we used proportional occurrence data based on taxon-specific visitation rates per observation date. In this way we were able to capture variation in the visiting insect communities among the two populations and the different survey dates. We used these data to calculate zero-adjusted Bray-Curtis dissimilarity matrices that were statistically tested in a PERMANOVA (9999 permutations) using the *adonis2* function in the 'vegan' package (Oksanen et al. 2022) with population as fixed factor. Ultimately, the mean, population-specific, insect visitor community was visualized in pie charts.

Diel patterns of scent emission

We tested whether the residuals of the total scent emission data met the assumption of normality using diagnostic plots and statistical tests ('DHARMa' package, Hartig et al. 2016). As the residuals showed no significant deviations from normality (Kolmogorov-Smirnov test: $p = 0.09$ for final model), and visual inspections of Q–Q plots indicated a good fit, we proceeded without log-transforming the response variable (total floral scent emission ng/flower/hour). To test for differences in total floral scent emission rate between the four times of day (dawn, day, dusk, night) we built six linear mixed models ('lmer', 'lme4' package ver. 35.1) with ng scent/flower/hour as response variable, time of day (dawn, day, dusk or night) as fixed factor, population (It4 or It10) as fixed or random factor, and seed family or plant individual alone or nested within start group as random factor (Table 1). Start group indicated what time of day a cycle was initiated for a specific group of plants. We included this factor to correct for any effect of exhaustion in the plants after several rounds of consecutive scent collection, as a measure to identify any decreased scent emission between the first and last scent collection round for the individual plants. The best fitted model was determined by conducting a likelihood ratio test where all seven models (six linear test models and one null model, Table 1) were compared in an ANOVA. The model AIC values were compared, and the model with at least 2 units AIC score less than the previous was determined as the best fitted model. To identify differences in floral scent composition between time of day, we performed Bray-Curtis zero-adjusted PERMANOVAs (*adonis2* function, 'vegan' package, 9999 permutations) using time of day (dawn, day, dusk, night) as fixed factor and scent compound composition as response variable, and subsequent non-metric multidimensional scaling (NMDS) plots (*metaMDS* function, 'vegan' package, $k = 2$, 200 tries) to visualize groups. These tests were performed on both the entire dataset and separately for the two populations.

Further, we compared day-time scent composition using the same method (PERMANOVA followed by NMDS plot) to identify floral scent divergence between the two populations. We used daytime collected samples in this test as previous studies investigating intraspecific variation in scent compound composition in *A. alpina* has been performed using samples collected during daytime (Petrén et al. 2021).

Table 1. Linear mixed models built using the 'lme4' package and lmer function in the statistical software R. Response variable for all models was 'Total Floral Scent Emission (ng/flower/hour)'. Time of day indicates collection time (dawn, day, dusk, night), population indicates individuals belonging to either It4 or It10, Seed family indicates individuals sharing maternal parent, Plant ID indicates individual plant identities, and Start group indicates what time of day a sampling bout was initiated for a group of plants (dawn, day, dusk, night). AIC in bold indicates best fitted model.

Model	Fixed factors	Random factors	AIC
1	Time of day, Population	Plant ID nested within Start group	1396.2
2	Time of day	Population, Plant ID nested within Start group	1379.2
3	Time of day, Population	Plant ID	**1368.2**
4	Time of day	Population, Plant ID	1373.9
5	Time of day	Population, Seed family nested within Start group	1404.4
6	Time of day	Seed family	1392.6
Null		Plant ID	

Effects of temperature on floral scent emission

To test for differences in total scent emissions caused by changes in temperature, we compared total floral scent emissions (ng/flower/hour) from *A. alpina* plants in cold day and night temperatures (5°C both day and night), to total scent emissions from It4 plants in warm day and night temperatures (18°C days and 16°C nights). Due to multicollinearity between explanatory variables, we collapsed time of day and temperature into a new variable (Time_temp; Day18, Day5, Night16, Night5). We used a linear model with ng/flower/hour as response variable and Time_temp as fixed factor and Plant ID as random factor. To investigate if compound composition differed between cold days and cold nights, and between the different temperatures, we performed a PERMANOVA (9999 permutations) with either time (cold days and nights) or the collapsed variable Time_temp as fixed factor and compound composition as response variable.

Tissue specific scent emission

We tested for differences in compound composition between floral tissues (petals PE, sepals SE, reproductive organs RP and whole flower WF) using PERMANOVA (*adonis2* function, 'vegan' package, 9999 permutations) followed by an NMDS plot (*metaMDS* function, 'vegan' package, k = 2, 200 tries). We used floral tissue as fixed factor and compound composition as response variable in the PERMANOVA. The test was performed on the entire dataset as well as on the two populations separately. To identify individual compounds that differed between tissues, we performed similarity percentage analyses (*SIMPER* function, 'vegan' package) on the two populations together.

Results

Pollinator exclusion experiment

Generally, plants that were permanently open or only accessible to pollinators during daytime (day open, Fig. 2a) produced the highest number of seeds per fruit, suggesting that they are primarily pollinated by diurnally active pollinators (Kruskal-Wallis, $\chi^2 = 60.3$, df = 7, $p < 0.001$). However, only in It4 the differences between treatments were statistically significant, although the patterns were similar in both populations (Fig. 2a). The higher altitude population It10 produced fewer seeds per fruit in all treatments except for the negative control (Fig. 2a), but proportionally more seeds per fruit in plants only accessible to nocturnal pollinators, indicating that plants in this population were visited by pollinators also at some point between 20:00 and 08:00 h.

Insect visitor community

We found a small ($R^2 = 0.06$) but significant difference between the two visitor communities (PERMANOVA, df = 1, F = 2.3, p = 0.01, Fig. 2b, Supporting information).

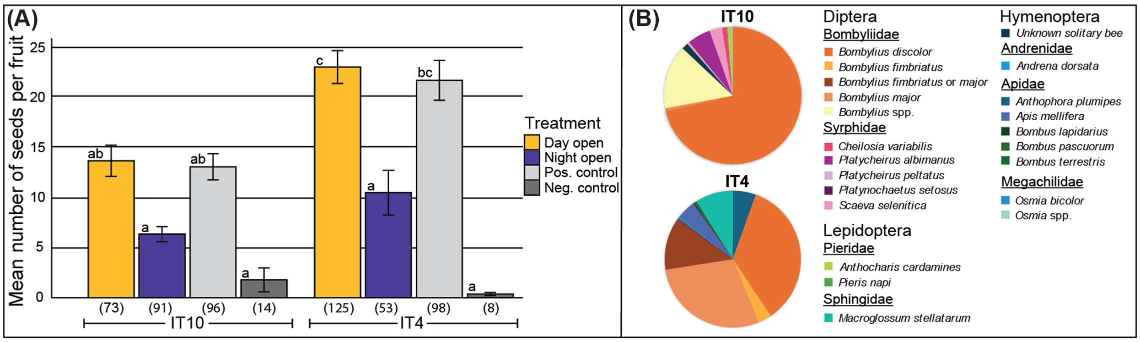

Figure 2. Mean number of seeds per fruit and insect communities of the two *Arabis alpina* populations. (a) Mean number of seeds produced per fruit and treatment in the two populations. Error bars indicate ± SE. Letters denote significant differences. Numbers in brackets below bars indicate sample size (number of fruits per treatment). (b) Mean insect communities (mean visits/flower/hour) in the two populations in 2022.

Both populations were dominated by different species of Bombyliid flies (Bombylidae). In It10, *Bombylius discolor* dominated the visits, while in It4 *B. discolor* too was a considerable part of the community but shared space with *B. major, B. major/fimbriatus* (combined due to uncertain identification) and *Macroglossum stellatarum* (Sphingidae, hummingbird hawkmoth) that was absent from It10. Other unique visitors in It10 included *Platycheirus albimanus* (Syrphidae) and *Anthocharis cardamines* (Pieridae), whereas *Apis mellifera* was only observed in It4. We observed muscoid flies visiting the flowers but these never interacted with the reproductive organs, and were hence excluded from all analyses and visual representations.

Diel patterns of scent emission and effect of temperature

We found that total scent emission rate was similar between the four times of day (dawn, day, dusk, night) in both populations (best fitted model, model 3, Table 1). The best fitted model (model 3, lowest AIC score) contained time of day and population as fixed factors and plant ID as random factor and was significantly different from the null model model (ANOVA, $p < 0.001$ (χ^2)). However, the overall lower emission rates from It4 plants (Fig. 3a) were responsible for this effect, as removing population completely from the model, time of day alone did not have the same influence total floral scent emission (ANOVA, $p = 0.14$ (χ^2)). No other model was significantly different from the null model. Further, start group had no influence on total scent emission (comparing model AIC with and without start group as random factor: ANOVA, $p = 1$ (χ^2), $\Delta AIC = 11$, Model 3 and 2, Table 1).

Further, we did not find any evidence of changes in compound composition between the different times of day for It10 (PERMANOVA, $df = 3$, $R^2 = 0.03$, $F = 0.5$, $p = 0.7$). In It4, we discovered a slight shift between dawn-day and dusk-night samples (PERMANOVA, $df = 3$, $R^2 = 0.1$, $F = 2.4$, $p = 0.03$, Fig. 3d), where the dusk-night scent composition included somewhat less variation among samples than dawn-day samples (Fig. 3d). Dusk-night samples also contained traces of benzenepropanol and 4-methoxybenzaldehyde, which the dawn-day samples did not. Running the test across both populations revealed no significant difference in compound composition between times of day (PERMANOVA, df= 3, $R^2 = 0.03$, $F = 1.5$, $p = 0.14$, Fig. 3b). Overall, our tests suggest that *A. alpina* emits similar amounts of scent of similar composition regardless of time of day. A list of detected compounds in both populations is available in Supporting information.

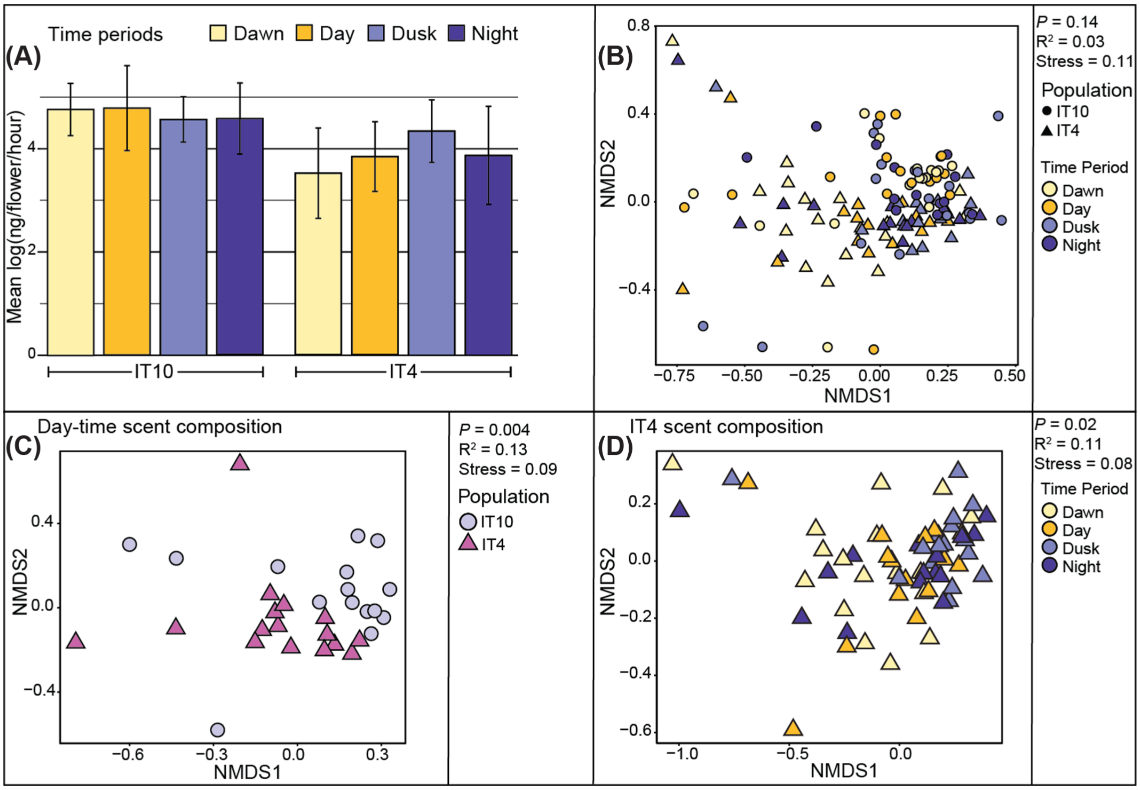

Figure 3. Floral scent emission rate and composition in the two *Arabis alpina* populations. (a) Mean log(nanograms/flower/hour) floral scent emission in It10 and It4 across the four different time periods. Error bars indicate ± SE. (b) Compound composition visualized in an NMDS plot of floral scents collected from both populations (It10: circles, It4: triangles) and the four different times of day. (c) Compound composition visualized in an NMDS plot of floral scents emitted from both populations during day-time. (d) Compound composition visualized in an NMDS plot of floral scents emitted from It4 plants during the four different times of day.

We found no evidence for decreased floral scent emission in cold compared to warm temperatures (ANOVA, linear model, df=3, $\chi^2=4.11$, p=0.24). Scent composition did not change between cold days and cold nights (PERMANOVA, df=1, $R^2=0.07$, F=0.5, p=0.6) or between the four combinations of time of day and temperature (Day18, Day5, Night16, Night5) (PERMANOVA, df=3, $R^2=0.07$, F=1, p=0.4).

Tissue-specific scent emission

Sepals, petals, and reproductive organs had distinct phytochemical profiles across both populations (PERMANOVA, df=3, $R^2=0.59$, F=24, p=0.01, Fig. 4a–b). This pattern was also true within populations ($\text{PERMANOVA}_{\text{IT4}}$, df=3, $R^2=0.56$, F=41, $p < 0.01$; $\text{PERMANOVA}_{\text{IT10}}$, df=3, $R^2=0.33$, F=7.9, p=0.02). Sepals (SE) were clearly separated for most samples from the rest of the tissues (Fig. 4b), due to them containing green leaf volatiles ((*Z*)-3-hexen-1-ol and 3-hexen-1-ol acetate) not present in the other tissues. In both populations, benzenoids and phenylpropanoids dominated petal emissions, with benzaldehyde being the most abundant compound making up 66% of the total compound abundance in It10. A list of all compounds detected in all tissues in both populations is available in Supporting information.

Population specific patterns

The dynamic headspace samples revealed a small difference in day-time scent compound composition between the two populations (PERMANOVA, df=1, $R^2=0.13$, F=4.7, p=0.004, Fig. 3b). Both populations contained large proportions of benzaldehyde, but It4 contained more phenylethyl acetate and benzyl acetate than It10, that instead had larger proportions of phenylethyl alcohol and phenylacetaldehyde compared to It4. Tissue specific scent emissions differed also between the two populations. For instance, It4 petals emitted ~ 11 times more phenylethyl acetate than It10 petals (SIMPER, $p_{\text{petals}}=0.001$) while both It10 petals (PE) and reproductive organs (RP) emitted on average ~3 times more phenylacetaldehyde compared with the corresponding It4 tissues (SIMPER, $p_{\text{petals}}=0.01$, $p_{\text{reproductive}}=0.03$).

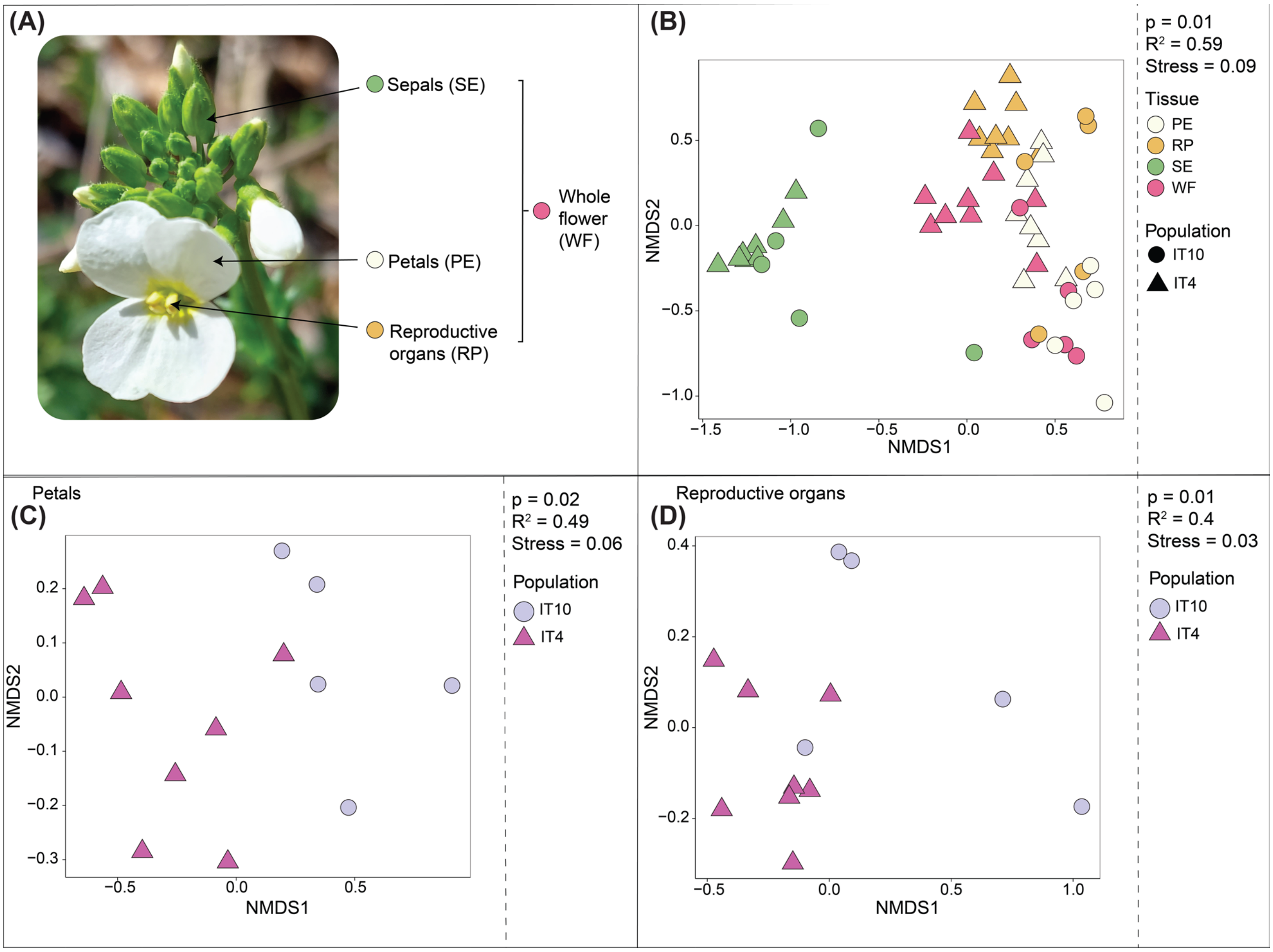

Figure 4. Tissue specific scent emissions from three floral structures: petals (PE), sepals (SE) and reproductive organs (RP), as well as whole flower (WF) sampled from *Arabis alpina*. (a) Schematic showing the sampled tissues. (b) NMDS plot visualizing compound composition of all tissues sampled from the two populations (It4: circles, It10: triangles). (c) Compound composition emitted from petals only, visualized in an NMDS plot. (d) Compound composition emitted from reproductive organs only, visualized in an NMDS plot.

Discussion

During the last decades, research on floral scent has revealed variation across multiple scales – among conspecific populations (Delle-Vedove et al. 2017, Petrén et al. 2021), among individuals within populations (Friberg et al. 2017, Petrén et al. 2021, Eisen et al. 2022a, Powers et al. 2022, Gfrerer et al. 2023), and among tissues within individual flowers (García et al. 2021). Similarly, floral scent has the potential to vary plastically in relation to the environment (Campbell et al. 2019, Höfer et al. 2022), and in synchrony with pollinators, potentially as a way to tailor the scent signals to periods of pollinator activity (Friberg et al. 2014, Chapurlat et al. 2018). This study evaluated small-scale spatial and temporal variation in the floral scent signal in an emerging eco-evolutionary model system, the perennial herb *A. alpina* (Wötzel et al. 2022), where previous studies have revealed genetically-determined floral scent variation across multiple European populations (Luizzi et al. 2021, Petrén et al. 2021, Thosteman et al. 2024). Our results revealed small-scale, tissue-specific variation in both floral scent composition, and in the community of floral visitors between two populations separated by only some 4 km. However, even though both populations, and It4 in particular, were predominantly pollinated during the day, their respective chemical signals within populations were very consistent across the diurnal cycle both in emission rate and in floral scent composition. These results suggest a lack of ecologic or metabolic cost(s) of emitting floral scent in this system, or that the cost of maintaining a system for up- or downregulation of scent outweighs the benefit of expressing a diel variation in floral scent signaling. Our results further indicate population-specific floral scent compositions at both geographic and tissue level, which potentially reflect a response to a varying local pollinator community.

Timing of pollination and insect community variation

Both populations, and It4 in particular, were predominantly visited by day-active insects (Fig. 2a), and the most common visitors in both populations were different bombyliid fly species (Fig. 2b). Although the two insect communities showed overlap in taxon identity, there were significant differences in community composition (Fig. 2b). This was exemplified by the unique presence of *Macroglossum stellatarum* and *Apis mellifera* in It4 and *Platycheirus albimanus* (Syrphidae) and *Anthocharis cardamines* (Pieridae) in It10. Although the habitats of the two populations have some similar features (e.g. deciduous tree shading, rocky or gravel rich habitat), they were located at different altitudes (Δ 800 m a.s.l.), and differed in other abiotic factors including vegetation density, grass cover and sun exposure. It is possible that taxon-specific differences between the two focal *A. alpina* populations can be derived from the difference in altitude (Malo and Baonza 2002, Brunet 2009, Gross et al. 2016, McCabe and Cobb 2021). Yet, unpublished data on the pollinator community composition in seven *A. alpina* populations (It4 and It10 included) in the same region and across different altitudes suggest that mechanisms other than altitude are driving the pollinator community variation (H. Thosteman et al., in prep.). Thus, further studies on how taxon-specific differences are related to altitudinal gradients in this system are needed to fully understand these dynamics. The absence of *M. stellatarum* in It10 was likely not caused by the difference in altitude between the populations, as this species was observed only 9 km east of It10 at 1600 m a.s.l. (similar altitude as It10), visiting *Vaccinium* sp. and *Calluna* sp. (H. Thosteman, pers. obs.).

In a parallel study (H. Thosteman et al., in prep.) the pollination efficiency of insect visitors to *A. alpina* flowers was evaluated across seven Apennine populations. Here, all visitors contributed to pollination to varying degrees. Although bombyliid flies were the most frequent visitors, they were neither the most effective pollinators nor the most important when accounting for visitation rate. Instead, when present, long-tongued bees (e.g. *Anthophora plumipes*) played the dominant role. In fact, in both It4 and It10, long-tongued bees contributed more to pollination than bombyliid flies, when accounting for both efficiency and visitation rate (H. Thosteman et al., in prep.). However, the pollination efficiency was generally evenly distributed across insects of several functional groups, further supporting a generalist pollination system of *A. alpina* populations in central Italy. In It10, plants produced seeds also when accessible only during night, indicating that successful pollination also occurred between 20:00 and 08:00 h. There are several examples of successful pollination occurring across all hours of the day in a single species, such as in *Hesperis matronalis* (Brassicaceae), where plants are visited by diurnal, crepuscular and nocturnal insects (Mitchell and Ankeny 2001, Majetic et al. 2009). Diurnal and nocturnal pollination could thus be true also for some *A. alpina* populations. Yet, the observed patterns in It10 may not necessarily reflect true nighttime pollination – pollination by insects that are active in the dark. On several occasions we arrived at the It10 population before 08:00 h and found sunlight hitting these focal plants, and day-flying insects already visiting the flowers exposed to morning sun early in the day. Thus, to fully refute or confirm the presence of nocturnal pollinators, nighttime pollinator observations should be included in future studies of *A. alpina*.

Diel patterns of scent emission and synchronization with pollinators

Contrary to our prediction, we found no evidence of up- or down-regulation of total scent emission rate in relation to peak pollinator activity in either population (Fig. 2a–3a). In both populations, plants emitted similar amounts of scent regardless of time of day and temperature. Interestingly, not even a dramatic temperature reduction to 5°C significantly affected scent emission. In many other species studied, floral scent emission is strongly affected by temperature (c.f. Jakobsen and Olsen 1994, Sagae et al. 2008, Cna'Ani et al. 2015, Fu et al. 2017), and such effects may both be attributed to an active reduction of emission rates and a passive response to e.g. reduced evaporation. In other systems, floral scent emission is less sensitive to changes in temperature (Theis et al. 2007, Friberg et al. 2014, Chapurlat et al. 2018), but instead follows pollinator activity or plant ontogeny. This study demonstrates that in *A. alpina,* floral scent tracks neither temperature, light, nor pollinator activity. Furthermore, previous work showed that floral scent in *A. alpina* was practically unresponsive to changes in nutrient and water availability (Luizzi et al. 2021). Together, these results indicate that floral scent emission of *A. alpina* plants is highly canalized with little to no variation in response to the studied abiotic environmental factors. The stable rates of floral scent emission could indicate a lack of metabolic or ecologic cost of floral scent production and/or emission, although assessing the production cost of phytochemicals can be difficult (Irwin et al. 2004). Often, the pathways involved in floral scent biosynthesis are part of other metabolic processes, such as growth regulation, phytohormone production and protection against abiotic factors such as UV-stress (Kabera et al. 2014). Thus, downregulation of floral scent may have negative side effects. Further, it may not be the production that is costly, but also the process of up- and downregulation itself could be subjected to trade-offs linked to gene expression (Lang et al. 2009). If the process of up- or downregulation of floral scents itself is costly, evolution of diel rhythms of scent emission should only occur when the benefits outweigh that cost. For instance, specialized plants may optimize attraction of noctuid moths, which almost exclusively rely on olfactory signals when foraging (Balkenius et al. 2006), while also evade herbivores during non-pollinator periods by up- and down-regulating scent emissions (Theis and Adler 2012). When such selective pressures to up- and downregulate scent emission are lacking, as in most *A. alpina* populations where herbivory is almost completely absent (unpubl. data), we may expect scent-emitting flowers to produce constant emission rates. Revealing the cost of floral scent production is one of the great challenges ahead for the field of pollination ecology.

Many floral scent compounds included in the pollinator attraction module could have a separate function as herbivore repellents (Pichersky and Gershenzon 2002, Irwin et al. 2004, Theis et al. 2007). In this study, we did not include herbivores as a potential variable for floral scent emission and composition, and using volatiles emitted from the flowers to repel enemies such as florivores could be a possible explanation for the relatively stable rates of scent emission also during nighttime in this species. In the studied populations we find very little evidence of leaf damage caused by herbivores, or damage to flowers caused by florivores or seed predators (unpublished data), although more substantial herbivory has been documented in other populations (Buckley et al. 2019). Thus, protection against antagonists may be a possible cause for the constant scent emissions. However, leaf defense volatiles and floral scent volatiles show very low levels of integration in *A. alpina* (Thosteman et al. 2024), and herbivore induced leaf defense volatiles are thus unlikely to affect the rates of floral scent emission. Further, the headspace volatiles documented in this experiment are almost exclusively aromatics, while terpenoid compounds that are typically associated with herbivore defense are emitted at only low rates in *A. alpina* (Pichersky and Gershenzon 2002). Conversely, many of the aromatic constituents of the *A. alpina* scent bouquet are common compounds known to attract pollinators across several orders (Andersson and Dobson 2003, Rachersberger et al. 2019), and floral aromatic emissions is unlikely to have an anti-herbivore evolutionary origin (Schiestl 2010). Still, detailed studies are needed to fully understand the bioactive function of the stable emissions of floral scent in this species.

Finally, we found no clear evidence of change in compound composition between the four times of day. There were patterns of slight shifts in scent composition between It4 dawn-day samples and It4 dusk-night samples (Fig. 3d), where dusk-night samples contained trace amounts of some compounds that were absent in the dawn-day bouquets. It is not unreasonable to imagine the presence of these compounds to have a distinct purpose, as insects are sometimes able to respond to minimal changes in the floral scent bouquet (Wright and Schiestl 2009). Yet, the trace compounds were only detected in a very limited number of samples, and we are thus unable to rule out methodological constraints, such as presence of compounds below detection level. Regardless, future studies on the bioactive properties of all compounds could provide further information on diel patterns of herbivore repellence or pollinator attraction in *A. alpina*.

Sources of floral scent variation

Our results suggest both quantitative and qualitative differences in the floral scent profiles of the two study populations. While this variation could be ascribed to differences in growth conditions (described above), our results are highly similar to those of previous studies of *A. alpina* (Petrén et al. 2021, Eisen et al. 2023), which suggests the observed population variation is not an environmental artifact.

The small, but significant, difference in floral scent bouquets emitted from the two populations during daytime (Fig. 3c) shows that consistent floral scent variation can be detected within a few kilometers. The among-population floral scent variation was localized to petals and reproductive organs (Fig. 4c–d), and the emission from these tissues were dominated by aromatic compounds. In It10, both reproductive and petal tissue were dominated by benzaldehyde, whereas, in It4, phenylethyl alcohol occurred at high abundance, in addition to benzaldehyde. Both these compounds are common constituents of floral scent also in other systems, and have been shown to be biologically active and important for pollinator attraction of e.g. Lepidoptera and Hymenoptera (Huber et al. 2005, Schiestl 2010).

In *A. alpina*, there was a particularly stark contrast between sepal scent composition and the other tissues (Fig. 4b), due to sepals containing several green leaf volatiles. It is interesting to note that sepals clearly separated from the whole flower samples (Fig. 4a), indicating that petals and reproductive organs were largely responsible for the composition of the total floral scent bouquet. Aromatics, which are often considered pollinator attracting compounds (García et al. 2021), were emitted from all three floral components: petals, sepals, and reproductive organs. However, the aromatic emissions from sepals were miniscule compared to those from petals and reproductive organs, suggesting that these compounds are important scent constituents in tissues involved in the pollinator attraction module of *A. alpina*.

Floral scents can also function as potential honest signals of floral rewards to pollinators (Knauer and Schiestl 2015, García et al. 2021). Phenylacetaldehyde, one of the aromatic compounds found in the *A. alpina* scent bouquet, was emitted in It10 to a larger extent than in It4. A previous study on *A. alpina* revealed this compound to be a potential honest signal for nectar volume, although the bouquet as a whole may not be (Eisen et al. 2023), and phenylacetaldehyde emission rate is positively correlated to nectar volume also in another crucifer, *Brassica rapa* (Knauer and Schiestl 2015), highlighting the importance of this compound. Our results, where phenylacetaldehyde is emitted in disparate amounts in the two populations, coupled with its potential as an honest signal, could indicate a history of divergent selection on this compound in the studied populations. Floral scent likely functions as an advertisement trait, and future studies should aim to relate divergence in compound composition between *A. alpina* populations to differences in pollinator communities, as has been shown in other systems at larger spatial scale (Wright and Schiestl 2009, Gross et al. 2016, Larue et al. 2016)

Conclusions

Our dissection of the scales at which floral scent varies within populations has shown distinct floral scent variation at very small spatial scale, but no evidence of diel variation in floral scent emission, as *A. alpina* emits very similar floral scent bouquets at similar emission rates during day and at night, regardless of temperature treatment or pollinator activity patterns. If floral scent is indeed an important trait for pollinator attraction, as indicated by a previous study at larger spatial scale (Petrén et al. 2021), the lack of synchronization between floral scent emission rate, composition and pollinator activity indicates that floral scent production and emission comes with low metabolic and/or ecological costs for *A. alpina* plants in our study populations.

We identify three major candidate drivers of floral scent diversification across spatial scales in *A. alpina*. Firstly,, floral scent could be neither costly nor under selection in this system, and the spatial variation in floral scent could then be generated by random genetic drift. Previous studies (Petrén et al. 2021, Thosteman et al. 2024) have indicated that Italian populations of quite different floral scent can be located in close geographic proximity, whereas populations of similar floral scent chemistry could be separated by many hundreds of kilometers. Hence, floral scent could be exclusively attributed to drift only if the colonization history was quite complex, and future genetic studies are needed to fully refute the neutral hypothesis for floral scent variation in this system. Alternatively, the major selection force acting on floral scent chemistry could be decoupled from pollinator attraction, but that would require a selection agent other than pollinator(s) to impose local and divergent selection on the floral scent traits. Putatively, plant antagonists could select upon the repellent and/or resistance function of floral volatiles (c.f. Theis 2006, Schiestl et al. 2011, Theis and Adler 2012, Knauer and Schiestl 2017), but in our studies we have identified very low levels of herbivory, florivory and seed predation in Italian populations of *A. alpina*. Finally, a previous study (Petrén et al. 2021) indicate a role of pollinators for larger-scale floral scent diversification in this system, and it is possible that local variation in pollinator interactions may drive floral scent differentiation also at local spatial scale.

This is the first study to formally identify the floral visiting communities of *A. alpina*, indicating that this species is indeed generalist-pollinated, in that it interacts with several different functional groups of pollinating insects. Furthermore, the communities of floral visitors varied at a small spatial scale, potentially setting the stage for local variation in the strength and direction of pollinator-mediated selection on floral scent chemistry. Thus, future studies should aim at determining to what extent the localized floral scent variation in *A. alpina* is indeed molded by pollinator-mediated selection from variable pollinator communities, while identifying alternative putative agents of selection on floral scent.

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Author contributions

Hanna Thosteman: Conceptualization (equal); Data curation (lead); Formal analysis (lead); Investigation (equal); Methodology (equal); Visualization (equal); Writing – original draft (lead); Writing – review and editing (lead). **Katherine Eisen**: Conceptualization (equal); Data curation (equal); Funding acquisition (equal); Investigation (equal); Methodology (equal); Supervision (equal); Validation (equal); Visualization (supporting); Writing – original draft (equal); Writing – review and editing (equal). **Clara McNaughton Montgomery**: Data curation (supporting); Investigation (supporting); Writing – original draft (supporting). **Xuefei Cheng**: Data curation (supporting); Investigation (supporting); Writing – original draft (supporting). **Loretta Pace**: Project administration (equal); Writing – original draft (supporting). **Magne Friberg**: Conceptualization (equal); Investigation (supporting); Methodology (equal); Project administration (lead); Supervision (lead); Validation (equal); Visualization (equal); Writing – original draft (equal); Writing – review and editing (equal).

Data availability statement

Data are available from the Dryad Digital Repository*:* https://doi.org/10.5061/dryad.dr7sqvb8z (Thosteman et al. 2025).

Supporting information

The Supporting information associated with this article is available with the online version.

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