

Honest floral signalling traits vary across and within populations in an insect-pollinated plant

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Abstract

1. In flowering plants that produce concealed rewards, pollinator foraging preferences may select for floral advertisement traits that are correlated with rewards. To date, studies have not focused on the potential for honest signals to vary across populations, which could occur due to differences in pollinator communities or plant mating system.
2. We tested for variation in honest signals across and within populations and mating systems in *Arabis alpina*, a broadly distributed arctic-alpine perennial herb that is visited by a variable community of insects. In a greenhouse common garden, we tested for correlations between corolla area, floral scent and nectar volume in 29 populations. In 12 field populations, we examined variation in pollen limitation and corolla area.
3. Across and within populations and mating systems, larger flowers generally produced more nectar. Total scent emission was not correlated with nectar production, but two compounds—phenylacetaldehyde and benzyl alcohol—may be honest signals in some populations. Corolla area was correlated with pollen limitation only across populations.
4. Our results suggest that honest signals may be similar across populations but may not result from contemporary direct selection on floral advertisements.

KEY WORDS

Arabis alpina, floral rewards, floral scent, natural selection, pollination, trait correlation, volatile organic compounds

1 | INTRODUCTION

Many floral traits have evolved in response to selection generated by pollinators (reviewed in Caruso et al., 2019). One way that pollinators generate selection on floral traits is through their decision-making while foraging. Pollinators may use floral advertisements such as colour, size and scent to assess the potential gain associated with

visiting a flower (Knauer & Schiestl, 2015; Raguso, 2004). As pollinators seek to maximize their rewards per visit, they are expected to generate selection on traits that function as 'honest signals' of a flower's reward status (Armbuster et al., 2005; Raguso, 2004; Stanton & Preston, 1988; Wright & Schiestl, 2009). In theory, the evolution of honest signalling is particularly likely when pollinators are limiting to plant reproduction in a population (Benzman, 2013), but other selective agents, such as herbivores, may also generate

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selection on floral traits and rewards and thus contribute to geographical variation in honest signals (Hoffmeister & Junker, 2017; Knauer & Schiestl, 2017; Schiestl et al., 2011).

Two floral traits that are not direct properties of floral rewards and are likely 'indirect' honest signals are flower size and floral scent. In systems where floral rewards are hidden, pollinators may learn to use visible morphological traits that are correlated with rewards as advertisements of reward status (Cresswell & Galen, 1991). Aspects of flower size such as corolla area and inflorescence number in species with highly aggregated flowers (e.g. Armbruster, 1991), which function as advertisement traits without affecting pollination efficiency, are often correlated with nectar volume (Cresswell & Galen, 1991; Fenster et al., 2006; Harder et al., 1985) and nectar sugar concentration (Plowright, 1981).

While floral scent can serve as a long-distance pollinator attractant in some systems (Dufay et al., 2003; Raguso & Willis, 2002; Roy & Raguso, 1997), it also can affect pollinator behaviour at short-range distances via tissue-specific scent emission (reviewed in García et al., 2021). Relative to other advertisement traits, scent may be particularly likely to be an effective honest signal for several reasons. First, pollinators can quickly learn to associate scent with a food source (reviewed in Wright & Schiestl, 2009), which may generate selection for scent emission in rewarding species. Second, non-rewarding species are frequently scentless (Wright & Schiestl, 2009) or emit variable scent which pollinators cannot associate with rewards (Salzmann et al., 2007). If scent production has metabolic (Gershenson, 1994; Vogel, 1983) or ecological costs (Kessler et al., 2013), then low or variable scent may reflect selection to reduce these costs where scent does not serve as an honest signal due to a lack of rewards. However, floral scent has only been demonstrated to function as an honest signal in one species to date, where pollinator behavioural assays under laboratory conditions indicated a preference for the honest signal (Knauer & Schiestl, 2015, 2017, but see Burdon et al., 2020). As such, it remains unknown whether scent primarily functions as a short-range pollinator attractant because it is an honest signal of floral reward (reviewed in García et al., 2021), or as a long-distance pollinator attractant (Dufay et al., 2003; Raguso & Willis, 2002; Roy & Raguso, 1997), as these functions may vary across systems, or even within a plant species that interacts with several types of pollinators.

The evolution of honest signals via selection on traits or trait correlations (e.g. Benítez-Vieyra et al., 2010, 2014) requires sufficient variance in relative fitness, which determines the opportunity for selection (Crow, 1958; Endler, 1986). Variance in relative fitness is partially determined by the degree of pollen limitation of female fitness, which reflects the extent to which pollination limits seed production (Knight et al., 2005). Pollen limitation often varies within species across populations (reviewed by Knight et al., 2005), and is related to variation in the strength of pollinator-mediated selection (Trunschke et al., 2017). Contemporary selection for honest signals (here, selection on the advertisement) should be stronger in populations that are more pollen limited, where the foraging behaviour of pollinators is likely to have a greater effect on plant fitness, although variation in pollen limitation does not always explain variation in pollinator-mediated selection (e.g. Sletvold & Ågren, 2014).

While a recent meta-analysis (Parachnowitsch et al., 2019) documented a trend for positive correlations between floral advertisement traits and floral rewards across 22 studies, examining variation in the relationship between signals, rewards and reproductive success both across and within populations (Armbruster, 1991) will extend our understanding of how honest signals function and evolve. Fundamentally, correlations across populations represent the outcome of evolutionary processes but do not directly indicate the potential for future evolution (Agrawal, 2020), as selection acts within populations and correlations can result from both adaptive and non-adaptive evolutionary processes. As such, patterns across populations may result from adaptive divergence that could be due to past or ongoing selection, while patterns within populations can indicate if a trait or trait correlation may be the target of contemporary selection. Across populations, correlations between traits and rewards or reproductive success could be the product of a 'universal fitness landscape' (Peiman & Robinson, 2017), wherein selection always favours the same type of trait value. Alternatively, they may result from selective correlations/covariance (Armbruster, 1991; Armbruster & Schwaegerle, 1996), if selection favours certain combinations of trait values. Within populations, selection for honest signals may vary if populations differ in the abundance or composition of the pollinator community (Gómez et al., 2009; Herrera, 1988) or plant mating systems (Goodwillie et al., 2005); both pollinator community and mating system variation can affect the availability of or dependence on pollinators. In particular, honest signals should be under stronger selection within populations where plants are more reliant on pollinators for reproduction (Benkman, 2013).

In this study, we examine correlations across and within populations to investigate whether corolla area and floral scent could function as honest signals of floral rewards and whether corolla area covaries with pollen limitation in an arctic-alpine herb, *Arabis alpina*. An emerging model system in chemical and molecular ecology (Wötzl et al., 2022), *A. alpina* exhibits substantial variation in corolla area, floral scent and reliance on pollinators (see Section 2.1), which enables testing for honest signals at within- and across-population scales. We predict that floral scent is an honest signal in *A. alpina* because there is evidence of pollinator-mediated selection on floral scent (Petrén, 2020) and lower scent emissions in self-compatible relative to self-incompatible populations (Petrén et al., 2021). In addition, the compound that was determined to function as an honest signal in another Brassicaceae species (phenylacetaldehyde; Knauer & Schiestl, 2015) is also emitted in all self-compatible populations measured to date, and some self-incompatible populations in Greece and central Italy (Petrén et al., 2021). We focused on nectar volume as a measure of floral reward here because while some visitors to *A. alpina* do forage for pollen, bombyliid flies forage exclusively for nectar and are the most abundant visitor in multiple (but not all) populations (H. Thosterman, C. Montgomery, S. Blackburn, A. Susheel, X. Cheng, H. Petrén, S. Boutsi, J. M. Halley, L. Pace, & M. Friberg, in prep.). To determine whether corolla area and floral scent are honest signals and whether the relationship between these traits varies across populations and mating systems, we quantified variation among and within 29 populations from across Europe in a greenhouse common garden experiment. We

predict stronger correlations between nectar reward and advertisement traits in populations that are self-incompatible and interpret correlations across and within populations as evidence for global and local selection, respectively (Figure 1). In addition, we estimated pollen limitation and its relationship with variation in corolla area across and within 12 natural self-incompatible populations. We expected negative correlations between pollen limitation and flower size across populations, if selection on flower size occurred in the past, and within populations, if there is present-day selection on flower size (Figure 1).

2 | MATERIALS AND METHODS

2.1 | Study system

Arabis alpina L. (Brassicaceae) is a perennial herb widely distributed in arctic-alpine mountainous environments in Europe, East Africa, Asia Minor and eastern North America (Koch et al., 2006; Wötzl et al., 2022). It occurs in rocky, disturbed habitats, often on scree

slopes, rock ledges or near small streams. Self-compatible populations in Scandinavia have a higher capacity for autonomous self-pollination, with shorter anther-stigma distances and inward facing anther orientation (Toräng et al., 2017), relative to self-incompatible populations in Spain and France, while populations in Greece and Italy are self-incompatible (Petrén et al., 2021; Tedder et al., 2011; Toräng et al., 2017). Floral scent is comprised primarily of aromatics, and varies among and within populations and mating systems, as does corolla area (Petrén et al., 2021)—generally, self-incompatible populations produce larger and more strongly scented flowers than self-compatible populations, but there is ample variation in both traits. Pollinator observations have been conducted in a small number of populations to date, where flowers are primarily visited by dipteran, hymenopteran and lepidopteran insects (Petrén, 2020), but detailed studies in multiple central-Italian populations indicate that bombyliid flies are often the most frequent floral visitors in this area, whereas other populations are more frequently visited by day-flying hawkmoths or bumble-bees (H. Thosterman, C. Montgomery, S. Blackburn, A. Susheel, X. Cheng, H. Petrén, S. Boutsi, J. M. Halley, L. Pace, & M. Friberg, in prep.).

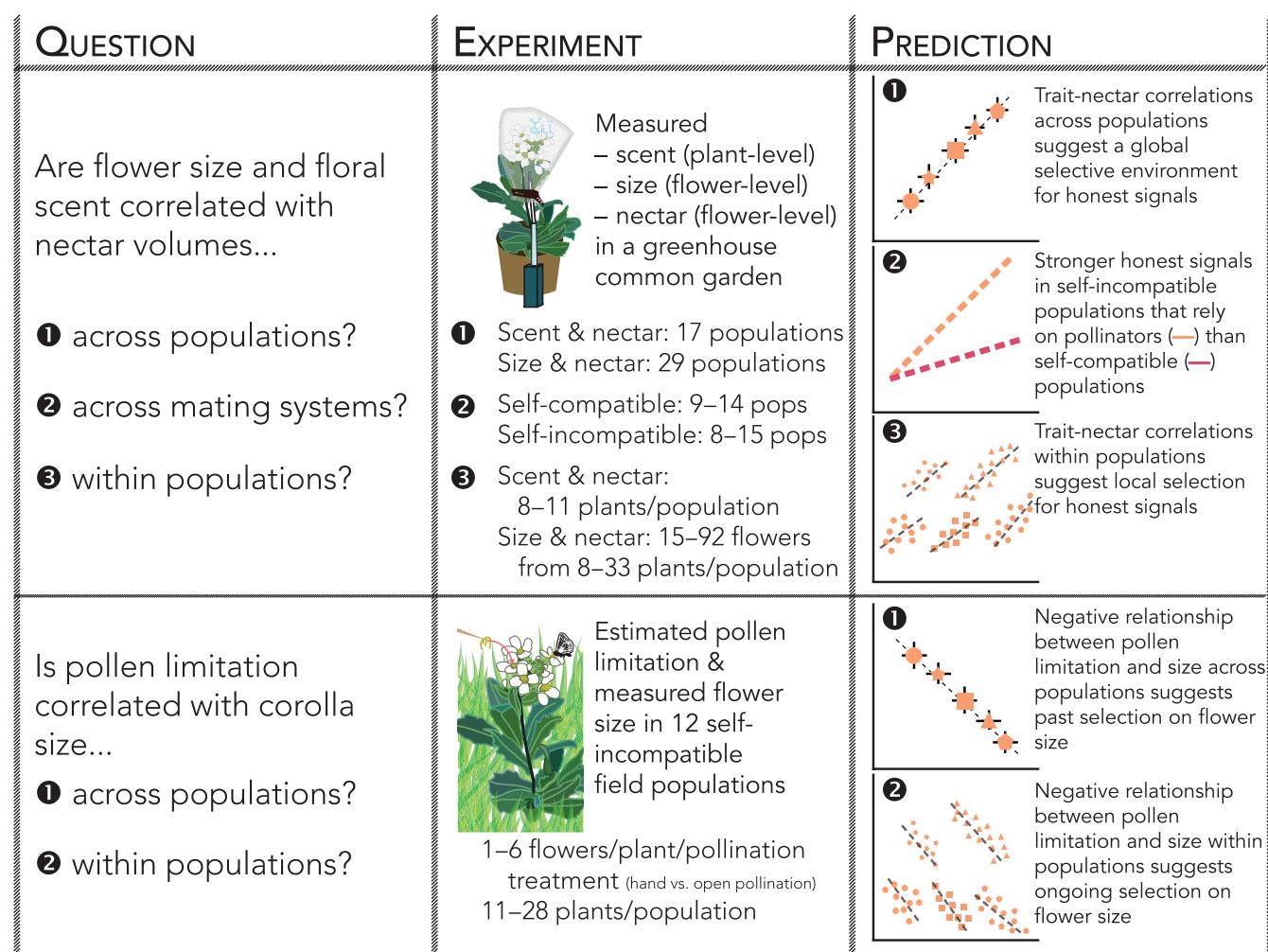


FIGURE 1 An overview of the questions, methods and expectations of the two main components of this study, a greenhouse common garden experiment to test for correlations between nectar rewards and potential honest signals both across and within populations, and a field study of variation in pollen limitation and flower size across and within populations.

2.2 | Greenhouse common garden methods

To assess correlations between corolla area, scent and nectar production, plants were grown in a greenhouse common garden from seeds collected in 29 populations from 6 countries: France (4 populations), Greece (3), Italy (12), Spain (1), Sweden (1) and Switzerland (8) (Table S1). For each population, we planted seeds from 15 open-pollinated seed families that were haphazardly selected from plants in the field; formally analysing variation across and within seed families (e.g. heritability or repeatability) was beyond the scope of this study. Ultimately, 2 plants from each of 15 seed families per population were grown beginning in September 2019. Details of plant germination and growth methods are provided in Supporting Information S2.

To estimate corolla area and nectar volume, we sampled a minimum of eight flowering individuals from a minimum of six seed families from each population (total $N=342$; sample sizes by population in Table S1). Between 1 and 6 flowers (mean: 2.8 flowers) were sampled on each plant (total $N=937$; sample sizes by population in Table S1), all of which had opened within 48 hours prior sampling; as such, all flowers were at the same phenological stage when the measurements were taken. Ninety-eight percent of nectar measurements were made across 24 sampling days between 25 February and 31 March 2020 ($N=990$ measurements, mean per day=41 flowers). The remaining measurements ($N=24$, 2% of all measurements) were taken across 9 days between 1 April and 15 April 2020 to increase the sample sizes of Greek populations that were slow to flower. Flowers were photographed next to millimetre paper using a microscope camera (Celestron Handheld Digital Microscope Pro) to estimate corolla area (Supplemental Methods, Figure S1). Nectar was collected from both spurs of these flowers using 0.5 or 1.0 μ L microcapillary tubes (Drummond Scientific Company, USA), which is a method that is suitable for flowers with small volumes of nectar (Morrant et al., 2009). Nectar volume was calculated from photographs of the microcapillary tubes using the same setup that was used to measure corolla area (Supplemental Methods, Figure S2). Out of the 937 total flowers sampled, no nectar was recorded from 184 flowers (19%). These zero values, which varied across populations (see Section 3), were retained in the dataset and were considered true biological zeros, as no liquid was visible in these flowers prior to sampling (S. Boutsi, pers. obs.).

2.3 | Scent methods

Floral volatile samples were collected using the dynamic headspace adsorption technique (Raguso & Pellmyr, 1998) between 3 November 2020 and 13 May 2021 on a haphazardly selected subset of the plants for which corolla area and nectar volume were measured in the greenhouse common garden (total $N=146$ plants; minimum $N_{\text{population}}=8$; Table S1). Previous work demonstrated that scent sampling in the greenhouse and in the field yield comparable results in *A. alpina* (Petrén, 2020; Petrén et al., 2021). Sampling

methods followed Petrén et al. (2021) and are described in detail in Supporting Information S2. We analyse total and specific compound scent emission rates of the 14 most frequently observed compounds in *A. alpina* which on average comprised 99.6% of the total scent emitted ($N=575$; Petrén et al., 2021).

2.4 | Field methods: Corolla area and pollen limitation

To assess variation in corolla area and pollen limitation in natural populations, plants were sampled in 12 self-incompatible populations in Italy and Greece in April–July 2019; six of these populations were included in the greenhouse common garden study (Table S1).

In each population, a maximum of 30 plants were haphazardly selected. Due to mortality and herbivory, sample sizes were reduced to between 11 and 28 plants per population (see Table S1 for exact sample sizes). Corolla area was measured for one representative flower per plant using the same method described above. To assess pollen limitation, 3–6 flowers per individual were hand-pollinated by saturating the stigma with pollen. Pollen was collected from individuals that were at least several meters away from the recipient. When hand-pollinations were performed, an equal number of flowers at a similar phenological stage on the same plant were designated as open-pollinated controls. These flowers were marked but not manipulated. Treatments on each plant were performed within a maximum period of 18 days. After approximately 9 weeks, the mature fruits from control and hand-pollinated flowers were collected and stored in individual paper envelopes. Some fruits from some Italian populations ripened unexpectedly early and opened before collection, leading to fruits with missing seeds. To estimate the total seed production of these fruits, we measured fruit length for all fruits from these populations. We then conducted population specific regressions between seed number and fruit length for the intact fruits from each population, to estimate the number of seeds in the open fruits (see Supplemental Methods, Table S3, Figure S3).

2.5 | Statistical analyses

All R code and data are available on GitHub and zenodo: <http://doi.org/10.5281/zenodo.8123411> (Eisen et al., 2023). All analyses were performed in R studio version 1.4.1717 using R version 4.1.0 (R Core Team, 2021). Linear mixed-effects models were run using the lmer function from the lme4 package (Bates et al., 2015).

2.6 | Analysis of honest signals in the greenhouse

To determine whether floral advertising traits could function as honest signals for floral rewards, we ran linear mixed-effects models. Although *A. alpina* plants often have multiple open flowers on a single inflorescence, leading to variation in the total size of the floral display, here we

focus on corolla area as a potential honest signal, as preliminary analyses indicated no relationships between display size and average nectar volume of a flower. All three variables (floral size, nectar volume and total scent emissions) were natural log-transformed for analysis. Because corolla area and total scent emission were weakly correlated ($r=0.22$, $p=0.002$), and because corolla area was measured at the flower level and on more flowers and plants than scent (see Table S1), we analysed the two traits separately. For the subset of plants for which scent, corolla area and nectar were measured, an analysis of size and total scent together produced qualitatively similar results (results not shown).

For corolla area, the unit of observation was individual flowers, as corolla area and nectar volume were both generally measured on multiple flowers per plant (described above). Given the relatively high proportion of flowers with zero nectar in this dataset (19%), a two-part hurdle model better fit this dataset, including corolla area in the model of the zeros was a better fit than a model without corolla area ($\Delta AIC = 32.1$). These models contained a random effect of plant ID nested within population.

For floral scent, the unit of observation was entire plants. Scent is expressed as emission rates (in ng/h) per flower (emission rate for a plant divided by the number of flowers open during the scent collection), and thus the response variable was the average per flower nectar volume per plant. For this dataset, a hurdle model with total scent emission was not an improvement over a hurdle model without total scent emission, and only 4% of plants in this dataset produced zero nectar. As such, the relationship between nectar and total scent was modelled using a one part linear mixed model, with the value +0.0001 added to all nectar measurements to enable natural log-transformation of the zeros. These models contained a random effect of population, except when we explicitly tested for variation across populations (see below).

We ran three sets of models for each potential honest signal. First, to test for an overall relationship between the potential signal and reward size, we ran a model with the signal as a fixed effect and plant ID and/or population as a random effect (described above), allowing for variation in both slopes and intercepts across populations. Second, we re-ran the model with an interaction between the signal and mating system. Third, we ran models with an interaction between the signal and population. For analyses of total scent and specific scent compounds, these were simple linear models. We report slopes ($\beta \pm SE$) estimated using the emtrends function from the EMMEANS package (Lenth, 2019), and correlations and associated p values were generated with the cor.test function.

Analysing variation in floral scent at the compound level is challenging because plants often produce many compounds via a small number of biosynthetic pathways such that each individual compound may not represent an independent trait value (Barkman, 2001; Eisen et al., 2022). Here we account for this by using a newly developed Bayesian reduced-rank regression approach (Opedal et al., 2022) to estimate the potential for scent as a composite trait to serve as an honest signal and to determine which compounds contribute to this composite trait. Briefly, this approach projects an original set of covariates onto a reduced set of composite variables that best explain variance in the response variable. To identify how specific compounds may then function as honest signals, we can project the estimated regression slopes

for the reduced rank covariates back onto the original variables. This was implemented using the HmSc 3.0 R package (Tikhonov et al., 2020) following a script developed by Opedal et al. (2022) which is available on GitHub (<https://github.com/kate-eisen/honest-signals>).

2.7 | Analysis of pollen limitation and variation in corolla area in the field

To determine whether populations of *A. alpina* are pollen-limited, we ran a linear mixed-effects model on the seed set data from each population. Number of seeds per fruit was modelled as a function of treatment (hand-pollinated vs open-pollinated), and plant ID was included as a random effect. We assessed the effect of the hand-pollination treatment using the anova function in the LMERTEST package (Kuznetsova et al., 2015) to perform type III F tests using the Kenward–Rogers approximation for the denominator degrees of freedom. We then used Tukey's honest significant difference tests to determine in which populations the treatments differed using the emmeans function (pairwise option) in the EMMEANS package (Lenth, 2019). A positive difference in seed set indicates that the hand-pollinated fruits set more seeds than the open-pollinated fruits, which is consistent with pollen limitation. A negative difference in seed set could occur if the hand-pollination process was detrimental to seed set (cf. Young & Young, 1992).

To determine whether pollen limitation is related to variation in corolla area across populations, we regressed population mean pollen limitation values on population mean corolla area. Pollen limitation was calculated as $(HP - C)/HP$, where HP and C are the mean seed set in the hand-pollinated and control flowers on a plant, respectively. To determine whether this relationship occurs within populations, we tested for a correlation between plant-level measures of pollen limitation and corolla area within each population. For both types of analyses, we present the slopes ($\beta \pm SE$) and correlations (r) as described above. Our dataset for pollen limitation on the plant level included negative values, which represent instances where hand-pollinated flowers set fewer seeds on average than control flowers. These instances may reflect ineffective hand-pollination, due to, for example, poor-quality pollen or damage to the flower when conducting the pollination, or a lack of pollen limitation in the population. Including the negative values, removing the negative values and truncating the negative values to zero (as negative pollen limitation can be interpreted as a lack of pollen limitation) all yielded qualitatively similar results (results not shown), and we report the results from the full dataset.

3 | RESULTS

3.1 | Trait variation across populations and mating systems

In our greenhouse common garden, we detected substantial variation in all three traits: over threefold variation across 29 populations in corolla area (range: 29.7–124.6 mm²; Table S4) [$LRT \chi^2(28) = 1163.6$,

$p < 0.001$], over 26-fold variation in nectar volume across 29 populations (0.004–0.112 μ L; Table S4) [LRT $\chi^2(28) = 225.4$, $p < 0.001$], and nearly 64-fold variation in total scent emissions across 17 populations (2.8–182.7 ng scent/flower/h; Table S4) [LRT $F(16) = 3.8$, $p < 0.001$]. For corolla area, approximately 70% of the total variance occurred at the population level, while 48% of the total variance in nectar volume occurred within plants (Table S5).

On average, flowers from self-incompatible populations were $\sim 2.3 \times$ larger, produced $\sim 2.5 \times$ more nectar and emitted $\sim 13 \times$ more scent emissions than flowers from self-compatible populations (Table S4).

3.2 | The relationship between floral advertisements and rewards

Across populations, floral size was positively correlated with nectar volume in all flowers measured ($r = 0.43$, $p < 0.001$, $N = 937$; Table 1). A hurdle model that included flower size was a better fit to this dataset than a null model ($\Delta AIC = 32.1$, see Section 2), as 19% of measured flowers contained zero nectar. For every 1% increase in corolla area, the log-odds of having non-zero nectar volume increases by about 3% (Figure 2a). Among those flowers that contained nectar, for every 1% increase in corolla area there was a 1.24% increase in nectar volume (Figure 2b). Mating system did not explain variation in nectar status (e.g. zero vs. non-zero nectar), and the relationship between corolla area and nectar volume among flowers with nectar did not differ between mating systems, with a 1% increase in corolla area corresponding to a 1.5% (self-incompatible plants) or 1.4% (self-compatible plants) increase in nectar volume. Populations varied in the number and proportion of flowers with zero nectar, with flowers from three self-compatible populations (AaSFH, Fr1 and Fr3) and one self-incompatible population (G3) more likely to contain zero nectar relative to all other populations (Table S1). After accounting for the relationship between corolla area and nectar status, five self-incompatible populations (G3, It10, It16, It18 and It6) were less likely to produce nectar relative to all other populations. Within populations, a non-zero slope between floral size and nectar volume among flowers with nectar was detected in 16 out of 29 total populations (Figure 2c). In these 16 populations, a 1% increase in corolla area was associated with between a 1.1% and a 3.7% increase in nectar volume, and correlations ranged from $r = 0.24$ to $r = 0.83$ ($p < 0.05$ in 14 of 16 populations; Table S6).

TABLE 1 Summary of the mixed models run on the full datasets (see Table S1 for sample sizes) with reward (log nectar volume) as the response variable and pollinator attraction traits (log corolla area, log total scent emission) as the explanatory variables. Binomial models tested the relationship between the explanatory variable and the presence or absence of nectar in the flower, while Gaussian models tested the relationship between the explanatory variable and quantitative variation in nectar.

Trait	Model type	$\beta \pm SE$	df	t	p
Corolla area	Binomial	2.7485 ± 0.4578	123.8781	6.003	<0.001
	Gaussian	1.2436 ± 0.1016			
Total scent emission	Gaussian	0.1212 ± 0.0711	11.8068	1.704	0.114

Across populations and mating systems, total scent emission was weakly correlated with nectar volume ($r = 0.18$, $p = 0.03$, $N = 154$ plants), but a 1% increase in total scent emission was not associated with an increase in nectar volume after controlling for population-level effects (Figure 3; Table 1), suggesting that the slight correlation between scent and nectar volume may be due to populations that vary in scent and nectar production. A hurdle model that included total scent was not a better fit to this dataset than a null model ($\Delta AIC = 2$ in the direction of the null model, see Section 2), and, as only 4% of these plants produced zero nectar, a hurdle model was not used to analyse this dataset. Out of 17 populations, total scent emission was positively associated ($\beta = 1.09$, $p = 0.001$; $r = 0.62$, $p = 0.053$) with nectar volume in one population, and negatively associated ($\beta = -0.46$, $p = 0.04$; $r = -0.42$, $p = 0.232$) with nectar volume in one population (Figure S4; Table S7).

Overall, one standard deviation change in the composite scent trait was associated with a 14% change in nectar volume (mean $\beta_{\text{scent}} = 0.14$ and credible interval: -0.078 , 0.4), and a relationship between the composite scent trait and nectar was fairly well supported statistically (89% posterior support). The strongest relationship between nectar and a single compound was with phenylacetaldehyde (β_{p} and credible interval: 0.07 , -0.02 to 0.24), which was emitted at higher rates (e.g. >5 ng/flower/h) in two populations where it was positively correlated ($r = 0.14$ and 0.44) with nectar volume (Figure S5). The compound with the second strongest relationship with nectar was benzyl alcohol (β_{b} and credible interval: -0.03 , -0.23 to 0.07), which was emitted at higher rates in three populations (Figure S5) with trends towards negative relationships between benzyl alcohol and nectar in two populations and a positive relationship in one population.

3.3 | Pollen limitation, corolla area and opportunity for selection in the field

Hand-pollinated flowers set more seeds than open-pollinated control flowers in four Greek and two Italian populations (Figure 4a; Table S8). Hand-pollination increased seed set from over 20% (population G7) to over 120% (population G9). The opportunity for selection (variance in relative female fitness) was higher and varied more among control flowers (median 0.70, range 0.39–1.44, $n = 12$) relative to hand-pollinated flowers (median 0.46, range 0.25–1.07, $n = 12$) (Table S8).

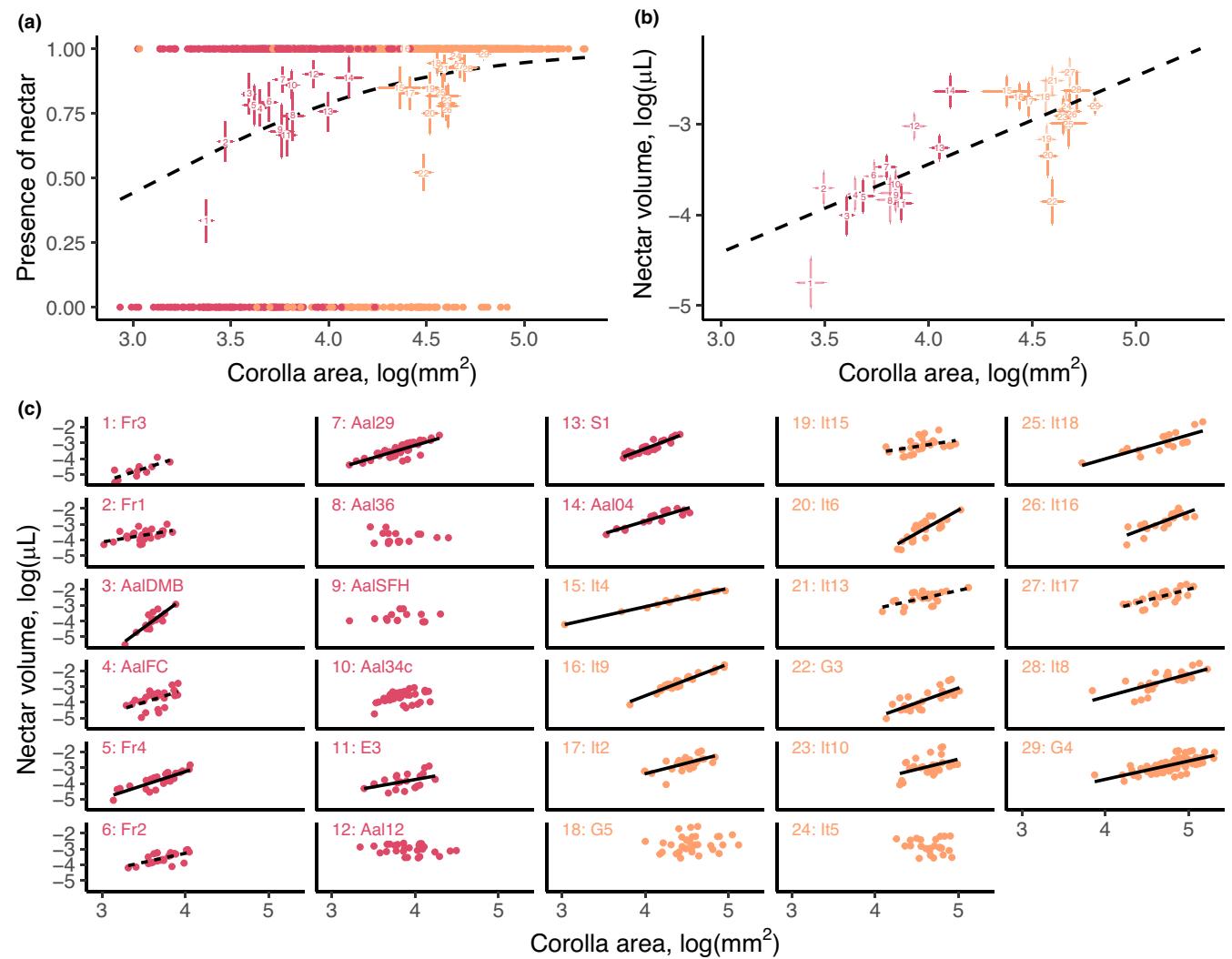


FIGURE 2 Across populations, the presence of nectar (0: a flower contains no nectar; 1: a flower contains nectar) (a) and nectar volumes in flowers with non-zero nectar (b) were positively associated with corolla area. Populations are numbered at the population means, with consistent numbering used across the panels, and crosses indicate standard errors for corolla area and nectar. Transparent error bars (in b) indicate populations where the within population relationship between nectar and corolla area was not significant. Within populations (c), corolla area was significantly associated with nectar volume in 16 of 29 populations (solid regression lines) and strongly positively associated (non-significant slope but $r \geq 0.2$) in seven additional populations (dashed regression lines). Points in panel (c) are predicted values from the linear mixed model that includes plant as a random effect. Self-compatible populations are represented in dark red, while self-incompatible populations are represented in orange.

Across populations, there was a trend towards a negative correlation ($r = -0.52$, $p = 0.08$, $N = 12$ populations) between mean pollen limitation and corolla area (Figure 4b). A 1 mm^2 increase in corolla area was associated with a trend towards a decrease in pollen limitation of 0.07 ($\beta \pm \text{SE} = -0.074 \pm 0.038$, $p = 0.080$).

Within populations, pollen limitation was correlated with corolla area in 2 out of 12 populations, and in opposite directions (Figure S6; Table S9).

4 | DISCUSSION

In a widely distributed perennial plant that receives visits from a diverse and variable community of insects, we found evidence both

across and within populations and mating systems that corolla area may serve as an honest signal of floral rewards. The correlation across populations could be due to a ridge or aligned peaks within a universal fitness landscape, although there are other non-adaptive explanations for this type of pattern, including pleiotropy and non-random dispersal among populations (Peiman & Robinson, 2017). The correlation within populations is consistent with local selection on honest signals (Figure 1). Flower size, which is correlated with nectar volume in many systems (reviewed in Parachnowitsch et al., 2019), may be particularly likely to be an honest signal if the size of floral display traits and the size of glands that secrete nectar are modular (Pélabon et al., 2012). In *Arabis alpina*, floral area could function as the key pollinator attractant, given that petals are bright white, but additional behavioural work with pollinators is needed (see below).

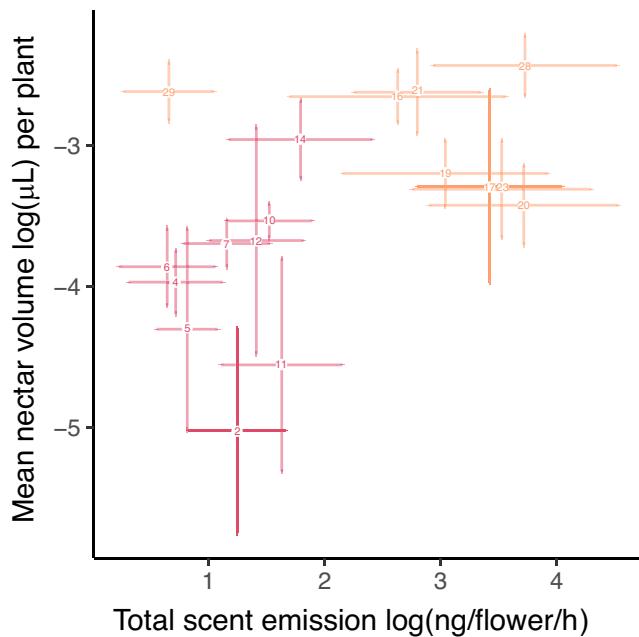


FIGURE 3 While overall total scent emission was weakly correlated with mean nectar volume, this relationship was not significant after accounting for population-level variation. Population numbering is consistent with Figure 1, and crosses indicate standard errors for total scent emission and nectar volume. Self-compatible populations are represented in dark red, while self-incompatible populations are represented in orange.

In contrast, total floral scent emission at the inflorescence level was not associated with nectar volume, but specific compounds may act as honest signals in a subset of populations. Here we discuss potential explanations for these patterns, and present ideas for additional research that would further our understanding of honest signals across scales.

Floral scent has been demonstrated to function as a long-distance pollinator attractant in multiple systems (Beker et al., 1989; Dufay et al., 2003), including plants that are visited by multiple insect taxa (Roy & Raguso, 1997). However, tissue-specific scent emission within flowers (reviewed in García et al., 2021) and evidence of pollinators learning scent cues (Wright & Schiestl, 2009) indicate that scent can mediate pollinator behaviour at short distances and, in one documented case, serve as an honest signal (Knauer & Schiestl, 2015). Using a newly developed Bayesian reduced-rank regression approach for dimensional reduction of floral scent data (Opedal et al., 2022), we found support for a relationship between nectar and a composite multivariate scent measure. The compounds identified by this analysis as potential honest signals—phenylacetaldehyde and benzyl alcohol—are known to attract pollinators to other Brassicaceae species (Gervasi & Schiestl, 2017; Majetic et al., 2007), and phenylacetaldehyde is an honest signal in one population of *Brassica rapa* (Knauer & Schiestl, 2015, 2017). The results from our studies of 17 populations of *A. alpina* suggest that such connections between particular compounds and the nectar reward may be local and dynamic. In fact, phenylacetaldehyde is not

emitted in all of the *A. alpina* populations studied to date (Petrén et al., 2021). Variation in the presence and/or abundance of phenylacetaldehyde may relate to variation in pollinator communities (Petrén, 2020; Thosteman et al., unpublished data) and indicates the potential for substantial variation in honest signalling across populations. Thus, the importance of floral scent as a long-distance attractant or a short-distance indicator of reward quantity may be spatially and temporally variable. However, assays of pollinator behaviour are needed to further assess whether total or compound-specific scent emissions function as pollinator attractants at shorter or longer distances in *A. alpina*.

There is ample opportunity for spatially variable pollinator-mediated selection acting on *A. alpina*. First, the variation in mating system spans populations that are largely autonomous, self-compatible but in need of pollinator vectors, and completely self-incompatible (Petrén et al., 2023; Toräng et al., 2015, 2017). Second, our study shows variation in pollen-limitation across self-incompatible populations, indicating variation in the opportunity for pollinator-mediated selection. We observed a negative relationship between pollen limitation and corolla area across populations, but corolla area was not related to pollen limitation within populations. The discord between these results highlights the importance of explicitly testing for relationships both across and within populations (Agrawal, 2020; Armbruster, 1991; Peiman & Robinson, 2017). Thus, our results suggest that the relationship observed across populations may reflect adaptive differentiation in response to past, not current, selection (e.g. Connell, 1980), although our measure of pollen limitation represents a single snapshot in time within the season, and variation in pollen limitation does not always relate to variation in pollinator-mediated selection (Sletvold & Ågren, 2014). The four- to five-fold variation in both pollen limitation, the slope of the relationship between corolla area and nectar, and the greater variation in flower size across relative to within populations all suggest that the selective environment for signal honesty could have varied across populations in the past, potentially due to variation in pollinator communities. Selection could vary due to variation in pollinator interaction intensity (Benkman, 2013), or the capacity for learning and discrimination (Cresswell & Galen, 1991; Wright & Schiestl, 2009). As a result, variation in pollinator communities could contribute to variation in honest signals, even if advertisement traits are not currently subjected to pollinator-mediated selection (e.g. Aigner, 2006).

Plant mating systems may contribute to trait variation without affecting the strength of honest signals. Specifically, in our greenhouse common garden, plants from self-incompatible populations had greater scent emissions and larger flowers with more nectar relative to plants from self-compatible populations (selective covariance; Armbruster & Schwaegerle, 1996), but trait associations were essentially identical across mating systems. There are multiple, non-mutually exclusive explanations for these patterns, although they could vary in the field if nectar rewards are curtailed by resource limitation. The production of less nectar, rather than zero nectar, in self-compatible plants could occur if nectar production is not particularly costly (Pélalon et al., 2012). Lower, but non-zero, nectar production

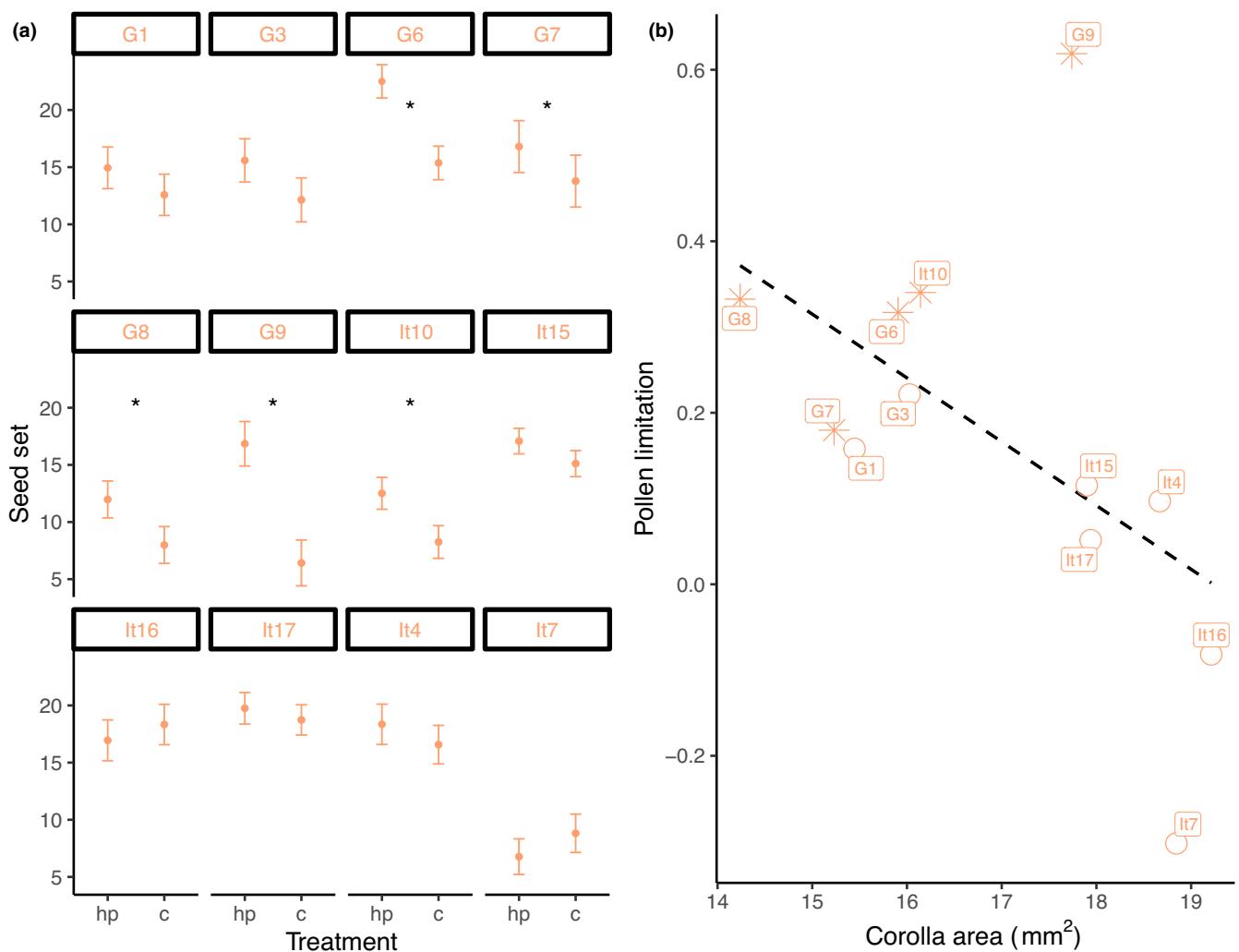


FIGURE 4 (a) Estimated marginal means with standard errors of seed set per fruit in the hand-pollination (Hp) and control (c) treatments. Asterisks indicate populations where the hand-pollination treatment set more seeds than the control treatment, which is consistent with pollen limitation. (b) Pollen limitation generally decreases with increasing corolla area. Populations plotted with asterisks are those with significant pollen limitation contrasts in panel (a). Pollen limitation was estimated solely in self-incompatible populations.

in self-compatible populations could also result from a constraint on the response to selection for lower nectar (Arnold, 1992; Ashman & Majetic, 2006), if nectar production is genetically correlated with another trait that is critical to reproduction. Finally, nectar production may be adaptive for attracting pollinators that induce geitonogamy in self-compatible populations in southern Europe where autonomous self-pollination is not uniformly high (Toräng et al., 2017).

Our study had three limitations. First, some of the variance in nectar volumes within plants could result from the challenges of measuring small nectar volumes (reviewed in Morrant et al., 2009). Future studies could determine whether a paper strip method, which can be used to estimate both sugar concentration (McKenna & Thomson, 1988) and nectar volume (Dungan et al., 2004), yields similarly high amounts of within-plant variance. Second, we measured scent at the level of the inflorescence, which meant that we were not able to relate variation in scent to variation in nectar at the flower level. Quantitative variation in scent (compound emission rates) is commonly measured at the inflorescence level, as sampling from

multiple open flowers may be either essential to detecting signal in weakly scented species (Ashman et al., 2005; Eisen et al., 2021) or it may be unavoidable if flowers are tightly clustered on inflorescences (Becklin et al., 2011; Gross et al., 2016). For both reasons, single flower dynamic headspace sampling is not feasible for *A. alpina*, but static headspace sampling of single cut flowers could be informative. Sampling dissected floral parts would indicate if compounds are emitted by specific tissues, which could serve to attract pollinators or repel antagonists at short distances (García et al., 2021). Third, we present data on nectar volume, which represents one potential axis of the rewards a plant may offer pollinators. Sugar concentration, while challenging to measure on small volumes as described above, could also influence pollinator behaviour. Pollen may represent an additional reward that was not considered here, but we hypothesize that pollen is not likely to be a primary reward in this system, given that bombyliid flies and day-flying hawkmoths were the most abundant visitors across multiple flowering seasons in multiple populations in central Italy and in one Greek population (G3; H. Thosterman,

C. Montgomery, S. Blackburn, A. Susheel, X. Cheng, H. Petrén, S. Boutsi, J. M. Halley, L. Pace, & M. Friberg, in prep.; Petrén, 2020).

Our study raises multiple avenues for future research that would provide further insight into the extent to which floral trait evolution is shaped by traits serving as honest signals of floral rewards. Potential relationships between advertisement and reward traits could be experimentally investigated in multiple ways. Artificial selection studies (Conner, 2003) would provide a way of determining if a species can respond to selection on honest signals, and if there is a genetic correlation between the traits (Pélabon et al., 2021). Alternatively, measuring honest signals in experimentally manipulated environments would indicate the extent to which honest signals are plastic, as there is strong evidence of plasticity in nectar (reviewed in Parachnowitsch et al., 2019) but mixed evidence for plasticity in scent (Burkle & Runyon, 2016; Campbell et al., 2019; Friberg et al., 2017; Luizzi et al., 2021). In addition, more studies that use learning bioassays to investigate how pollinators, and potentially antagonists such as herbivores, interact with honest and dishonest signals will provide further evidence for this potential driver of floral trait evolution (Knauer & Schiestl, 2015). Further investigations of how patterns vary across levels of biological organization will increase our understanding of the scale dependency of trade-offs and trait correlations (Agrawal, 2020).

AUTHOR CONTRIBUTIONS

Sotiria Boutsi, Hanna Thosterman, Hampus Petrén and Magne Friberg collected the greenhouse data, and Sotiria Boutsi and Hampus Petrén collected the field data with support from John M. Halley and Loretta Pace. Katherine E. Eisen analysed the data with input from Magne Friberg. Katherine E. Eisen wrote the manuscript, and all authors contributed substantially to revisions.

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CONFLICT OF INTEREST STATEMENT

None of the authors have a conflict of interest.

DATA AVAILABILITY STATEMENT

All data and code are available via GitHub and zenodo <http://doi.org/10.5281/zenodo.8123411> (Eisen et al., 2023).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. An example of the photographs used to measure corolla area. Measurements of petal length (blue arrow) and petal width (orange arrow) were used to calculate corolla area. The paper included in the images is millimeter paper.

Figure S2. An example of the photographs used to estimate nectar volume, indicated by the blue arrow. The paper included in the images is millimeter paper.

Table S1. Identifying information for the source populations used in the greenhouse common garden and the field measurements of pollen limitation.

Table S2. Volatile organic compounds (VOCs) that were summed to create the measure of total scent emission analysed, based on results of Petrén et al. (2021) (see main text). RT = retention time, minutes.

Table S3. Parameters for the linear mixed models used to estimate population-specific relationships between seed set and fruit length.

Table S4. Trait means (\pm SE) measured in the greenhouse. Sample sizes (N) given are the total number of plants/total number of flowers measured per population for the flower-level traits (nectar volume, corolla area), and as the number of plants for plant-level traits (scent emission rate). Scent was measured at the plant level, corolla area and nectar volume were measured at the flower level. Units are: Corolla area: sq mm, nectar volume: μ L, and scent: ng scent/flower/h

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