

# Local adaptation to hummingbirds and bees in *Salvia stachydifolia*: insights into pollinator shifts in a Southern Andean sage

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• **Background and Aims** Differences among populations in pollinator assemblages can lead to local adaptation mosaics in which plants evolve different floral morphologies and attractive traits. Mountain habitats may promote local adaptation because of differences in environmental conditions with altitude, causing changes in pollinators, and because mountaintops can act as isolated habitats. We studied if the differences in floral shape, size and nectar traits in *Salvia stachydifolia* can be attributed to variations in the relative contribution of hummingbirds and insects.

• **Methods** We studied eight populations of *S. stachydifolia* in natural and under common garden conditions, to assess whether population differences have a genetic component. We recorded pollinators, their behaviour and visitation rates, and characterized pollinator assemblages. In addition, we measured nectar volume and concentration, and collected flowers to describe floral shape and size variation using geometric morphometric methods. We then applied an unsupervised learning algorithm to identify ecotypes based on morphometric traits. Finally, we explored whether populations with different pollinator assemblages had different climatic and/or elevation preferences.

• **Key Results** We found that variation in the identity of the main pollinators was associated with differences among populations in all traits, as expected under a local adaptation scenario. These differences persisted in the common garden, suggesting that they were not due to phenotypic plasticity. We found *S. stachydifolia* populations were pollinated either by bees, by hummingbirds or had mixed pollination. We identified two ecotypes that correspond to the identity of the main pollinator guilds, irrespective of climate or altitude.

• **Conclusions** Variation in *S. stachydifolia* floral traits did not follow any evident association with bioclimatic factors, suggesting that populations may have diverged as the product of historical isolation on mountaintops. We suggest that differences among populations point to incipient speciation and an ongoing pollinator shift.

**Key words:** Bees, floral traits, geometric morphometrics, hummingbirds, local adaptation, mixed pollination, mountain habitats, nectar, pollinator ecotypes.

## INTRODUCTION

Geographical differences in pollinator assemblages can lead to local adaptation mosaics, where plants diverge in their floral traits (Johnson *et al.*, 2006; van der Niet *et al.*, 2014). This was first noted by Grant and Grant (1965), who suggested that ‘pollinator climates’ were as important in shaping floral phenotypes as abiotic factors were in shaping vegetative phenotypes (Moré *et al.*, 2020). Geographical mosaics of divergent selection can result in pollination ecotypes, i.e. plant populations morphologically distinct due to local adaptation (e.g. Parker *et al.*, 2018). Furthermore, this is predicted by the ecological speciation theory, as divergence in pollinator-related traits can lead to reproductive isolation (Schluter, 2001; Rundle and Nosil, 2005; van der Niet *et al.*, 2014). Even though some plant species are pollinated by both bees and hummingbirds (e.g. Barrionuevo *et al.*, 2021), there are few reports of geographical mosaics involving populations locally adapted to these two pollinators, particularly in the tropics. This absence is intriguing

because transitions from bee to hummingbird pollination are common in New World plant clades (Thomson and Wilson, 2008; Abrahamczyk and Renner, 2015). Environmental conditions, such as changes in pollinator effectiveness with altitude, have been suggested as drivers for these shifts (Cruden, 1972; Dellinger *et al.*, 2021, 2023).

Shifts between bee and hummingbird pollination in the Neotropics have been widely studied, but mainly from a macroevolutionary perspective (e.g. Abrahamczyk and Renner, 2015; Lagomarsino *et al.*, 2017; Serrano-Serrano *et al.*, 2017). Hummingbird and bee pollination are easily recognizable by the ‘floral syndrome’ they are associated with: hummingbird-pollinated plants tend to have flowers with large, red tubular corollas, with no scent and abundant dilute nectar, while bee-pollinated flowers show varied architectures and colours, and moderate amounts of concentrated nectar (Fenster *et al.*, 2004). These differences raise the question of how evolutionary shifts in pollination from one type of pollinator to another can occur

(Thomson and Wilson, 2008; Dellinger *et al.*, 2021), given that stable bimodal pollination seems unlikely due to the trade-off between adaptations for bee and hummingbird pollination (Castellanos *et al.*, 2004; Dellinger, 2020). However, few plant species have detailed pollinator records across their entire geographical range, which suggests that bimodal or locally adapted populations may be more widespread than previously thought. Geographical differences in extrinsic environmental factors may be crucial in pollinator shifts (Thomson and Wilson, 2008), as they may promote a change in the most efficient pollinator. Thomson and Wilson's idea can be encapsulated in the 'vortex metaphor', which describes the interaction between genetic processes and ecological factors. Each vortex represents a stabilizing selection regime on a set of phenotypes, corresponding to a particular pollination syndrome, i.e. the bee or hummingbird pollination. A pollination shift occurs when a new pollinator disrupts the stabilizing selection regime of the ancestral vortex.

Several floral traits that are targets of pollinator-mediated selection play a crucial role in understanding pollinator shifts. For example, traits in some hummingbird-pollinated flowers such as anther exertion, narrow corolla tubes and reduced flower structures that could act as landing platforms may be adaptations to prevent visits by bees or other insects (Castellanos *et al.*, 2004). Increased nectar volume and changes in corolla colour are also associated with hummingbird pollination (Bradshaw and Schemske, 2003; Wilson *et al.*, 2006; Cardona *et al.*, 2020). These examples indicate that requirements of mechanical fit and attractiveness for new pollinators require multivariate changes in the floral phenotype. Methods such as geometric morphometrics have been used to study local adaptation to different pollinator assemblages in Brassicaceae (e.g. Gómez *et al.*, 2009; Gómez and Perfectti, 2010), and explore pollinator shifts at a macroevolutionary scale in *Iochroma* (Smith and Kriebel, 2018), *Salvia* (Benitez-Vieyra *et al.*, 2019; Kriebel *et al.*, 2020) and *Meriania* (Dellinger *et al.*, 2019). These methods allow the study of floral morphology as a cohesive whole, enabling the interpretation, visualization and quantification of variation patterns (Zelditch *et al.*, 2012).

Mountain habitats offer an ideal location to study local adaptation to different pollinators. This is because altitudinal changes in environmental conditions may result in pollinator replacement from lowlands to mountaintops. At high elevations, bees may not be as efficient in pollination as endotherms such as hummingbirds, due to low temperatures and other environmental factors, such as mist (Cruden, 1972; Dellinger *et al.*, 2021, 2023). Additionally, mountaintops may function as isolated 'island' habitats, which may differ in pollinator faunas due to historical processes and local environmental heterogeneity (Perrigo *et al.*, 2020). In both cases, mountains may provide favourable scenarios for increasing and maintaining diversity.

In this work, we studied local adaptation of *Salvia stachydifolia*, an Andean species, to bee and hummingbird pollination. Previous research had classified this species as bee-pollinated based on its floral features (Wester and Claßen-Bockhoff, 2011), but recent observations have recorded mixed visits by bees, hummingbirds and bee-flies (Barrionuevo *et al.*, 2021) or only by bees (Saravia *et al.*, 2023). Taxonomic treatments of this species (Wood, 2007; O'Leary and Moroni, 2016) reported a wide variation in

flower size along its natural range in Bolivia and Argentina. This was confirmed by inspection of herbarium vouchers (J.V.I., unpubl. res.). Divergent selection may result in pollination ecotypes in *S. stachydifolia* because it grows in restricted island-like areas with a subtropical humid highland climate, isolated by dry valleys. *Salvia stachydifolia* belongs to the subgenus *Calosphace*, which includes ~600 species pollinated by bees or hummingbirds (Wester and Claßen-Bockhoff, 2011; Fragoso-Martínez *et al.*, 2018). Even though there are disagreements about the ancestral reconstruction of pollination shifts (Fragoso-Martínez *et al.*, 2018; Kriebel *et al.*, 2019; Sazatornil *et al.*, 2023), all studies have highlighted that shifts between bee and hummingbird pollination are particularly common in this clade.

Our goal here is to determine if differences in the floral shape, size and nectar traits of *S. stachydifolia* across different populations are due to variations in pollinator assemblages. To do this, we recorded pollinator visits and behaviour in eight geographically isolated populations. We used geometric morphometric methodologies to compare floral shape and size, and analysed differences in nectar volume and concentration. We conducted the same comparisons in a common garden setting to determine if population differences have a genetic component. Previous research has shown that corolla shape differs between bee- and hummingbird-pollinated species in *Calosphace* (Benitez-Vieyra *et al.*, 2019; Kriebel *et al.*, 2020), which can be explained by the distinctive behaviour of these pollinators when foraging: while bees land on certain flower structures and have a strong physical contact with flowers, hummingbirds hover around the flower with no need for a landing platform (Benitez-Vieyra *et al.*, 2019). Nectar also varies among *Salvia* species (Wester and Claßen-Bockhoff, 2011), with low nectar concentration discouraging bee visits, and probably acting as an anti-bee adaptation in hummingbird flowers (see Gegear *et al.*, 2017). In addition, we used an unsupervised learning algorithm on geometric morphometric data to identify plant ecotypes without prior knowledge of population identity (Rubini Pisano *et al.*, 2019). Finally, we examined the climatic and elevation characteristics of the study populations and additional records of *S. stachydifolia* occurrences.

## MATERIALS AND METHODS

### *Study system*

*Salvia stachydifolia* Benth. (Lamiaceae) is a perennial herb that grows up to 1–2 m tall. Inflorescences consist of terminal panicles, bearing ten or more flowers per verticillaster. The flowers have a typical bilabiate corolla shape with a lower corolla lip longer than the upper. *Salvia stachydifolia* is partially protandrous and self-compatible, but its reproduction depends mainly on pollinator activity. Concentrated nectar is produced in small amounts and accumulates at the base of the corolla (Barrionuevo *et al.*, 2021). Pollen is delivered by a staminal lever mechanism, as in most *Salvia* species. Bees, flies and hummingbirds were observed to visit the flowers in a southern population (Barrionuevo *et al.*, 2021), but only bees were recorded in a population from the northern tip of its distribution (Saravia *et al.*, 2023).

*Salvia stachydifolia* grows in open grasslands in the Southern Andean Yungas spanning from central Bolivia to NW Argentina at altitudes ranging from 1400 to 3500 m a.s.l. The Yungas are montane cloud-forests resulting from orographic rains, with a discontinuous distribution on the eastern slopes of mountain chains (Cabrerá, 1976). Rains are concentrated within 5–6 months in summer, and during colder months, fog partly compensates for the lack of rainfall (Brown et al., 2002). Within the Yungas, *S. stachydifolia* occurs in areas with a subtropical highland climate, usually with mist, growing on farm field borders, montane grasslands (Wood, 2007; O’Leary and Moroni, 2016) and occasionally in humid forest relicts inside dry valleys. It flowers in summer between January and March, with the flowering peak in February. Aerial parts of the plant die during winter when temperatures fall below 0 °C and regrow each spring from tuberous roots.

We conducted field observations between January and February in 2019 and 2020 in eight populations located in NW Argentina, including Río Singuil, Pozo de Piedra, Minas Capillitas, Tañí del Valle, Altos de Medina, Cuesta del Obispo, Lagunas de Yala and Rodeo Pampa (Fig. 1A; Supplementary Data Table S1). In each population, we extracted cuttings of ten plants to grow in a common garden located at the Instituto Multidisciplinario de Biología Vegetal (Córdoba, Argentina). Irrigation at the common garden was adjusted to mimic the average monthly rainfall of Tañí del Valle, with mist being provided three times per day during summer and one event per day during winter. However, the temperature in the common garden was that of Córdoba city, with a mean of 24.3 °C in the hottest month (January) and 10.8 °C in the coldest one (July). These temperatures were 3–10 °C higher than in any natural population of *S. stachydifolia*. We performed all measurements in common garden plants at least 12 months after plant establishment.

#### Floral visitors

To characterize pollinator assemblages in each population of *S. stachydifolia*, we conducted focal observations on floral visitors and recorded their visitation rates. We performed these observations in four different patches within each population, with each patch containing 50–200 flowers. In total, we observed floral visitors for 142 h, ranging from 13 to 27 h per population. We quantified visitation rates during four times: (1) morning (0800–0900 h), (2) midday (1100–1200 h), (3) afternoon (1500–1600 h) and (4) evening (1800–1900 h). These time periods were selected based on prior observations of bee and hummingbird activity (Barrionuevo et al., 2021).

We only recorded visitors that physically touched the anthers or the stigma, excluding nectar thieves. Thus, we considered all recorded visitors as potential pollinators (for brief ‘pollinators’ in the following). We visited five populations in January–February 2019 and four additional populations in February 2020, during the flowering peak of *S. stachydifolia*. Two populations were visited twice: Minas Capillitas and Pozo de Piedra. We conducted an additional 4 h of observations during the second visit. The data collected during the second visit were included in the analyses, as the identity and relative frequency of the main pollinator guilds remained stable (Supplementary Data Table S2).

#### Nectar

We harvested five first-day flowers from each of eight to 11 plants in natural populations ( $n = 356$  flowers, 76 plants), and the same number from five to nine plants per population in the common garden ( $n = 271$  flowers, 57 plants). To ensure nectar accumulation, we bagged the buds the day before data collection to avoid animal visits. We collected nectar between 1100 and 1200 h on the following day and measured nectar volume using 5- $\mu$ L calibrated microcaps (BLAUBRAND®) and nectar concentration using hand refractometers (0–32 and 28–62 Brix %, Atago®). To test for differences in nectar volume, we applied generalized linear mixed-effects models with Gamma error distribution, while to test for differences in nectar concentration, we constructed linear mixed-effects models with Gaussian error distribution. We treated plant identity as a random effect and population as a fixed effect in those models. To build the models, we used the *glmer* function from the *lme4* package (Bates et al., 2015) (Bates et al., 2015). To test for pairwise differences between populations, we estimated Bonferroni-adjusted marginal means obtained through the *emmeans* function from the *emmeans* R package (Lenth, 2022). We conducted all the analyses in R 4.2.1 software (R Core Team, 2022). We analysed the data from the common garden separately from the data from the natural populations.

#### Floral morphology

To examine variation in floral shape among populations, we collected on average three flowers per individual in 30 plants per population in the field ( $n = 787$  flowers, 269 plants) and three flowers per individual in five to ten plants per population in the common garden ( $n = 173$  flowers, 58 plants). We preserved these flowers in 70 % ethanol until diaphanization and then photographed the corollas and the attached anthers in lateral view, together with a reference scale, using a Nikon D5300 digital camera with Micro Nikkor 105-mm lens and back-light illumination. To quantitatively assess the shape of corolla and attached stamens, we used landmark-based geometric morphometrics, where landmarks are the coordinates of discrete loci that are homologous in all individuals (Zelditch et al., 2012). We used ten landmarks (Supplementary Data Fig. S1): insertion of corolla tube into the calyx, tip of upper lip, insertion of anther into the filament, insertion of the stamen filament into the corolla tube, corolla mouth, and constriction between middle and lateral lobes of lower lip. The remaining three landmarks were points of maximum curvature of the corolla. Additionally, we obtained 42 equidistantly spaced semilandmarks from three curves that describe corolla contour and extended (1) from the insertion of the corolla tube to the tip of upper lip, (2) from the insertion of the corolla tube to the tip of the lower lip, and (3) along the lower lip margin in lateral view (Fig. S1). We obtained landmark and semilandmark coordinates using the *StereoMorph* R package (Olsen and Westneat, 2015).

We performed geometric morphometric analyses using the *geomorph* R package (Adams et al., 2022). We first applied a generalized Procrustes analysis on the landmark coordinates to remove size and position effects. This analysis minimizes the squared Euclidean distances between homologous landmarks (Zelditch et al., 2012) and preserves only shape information in



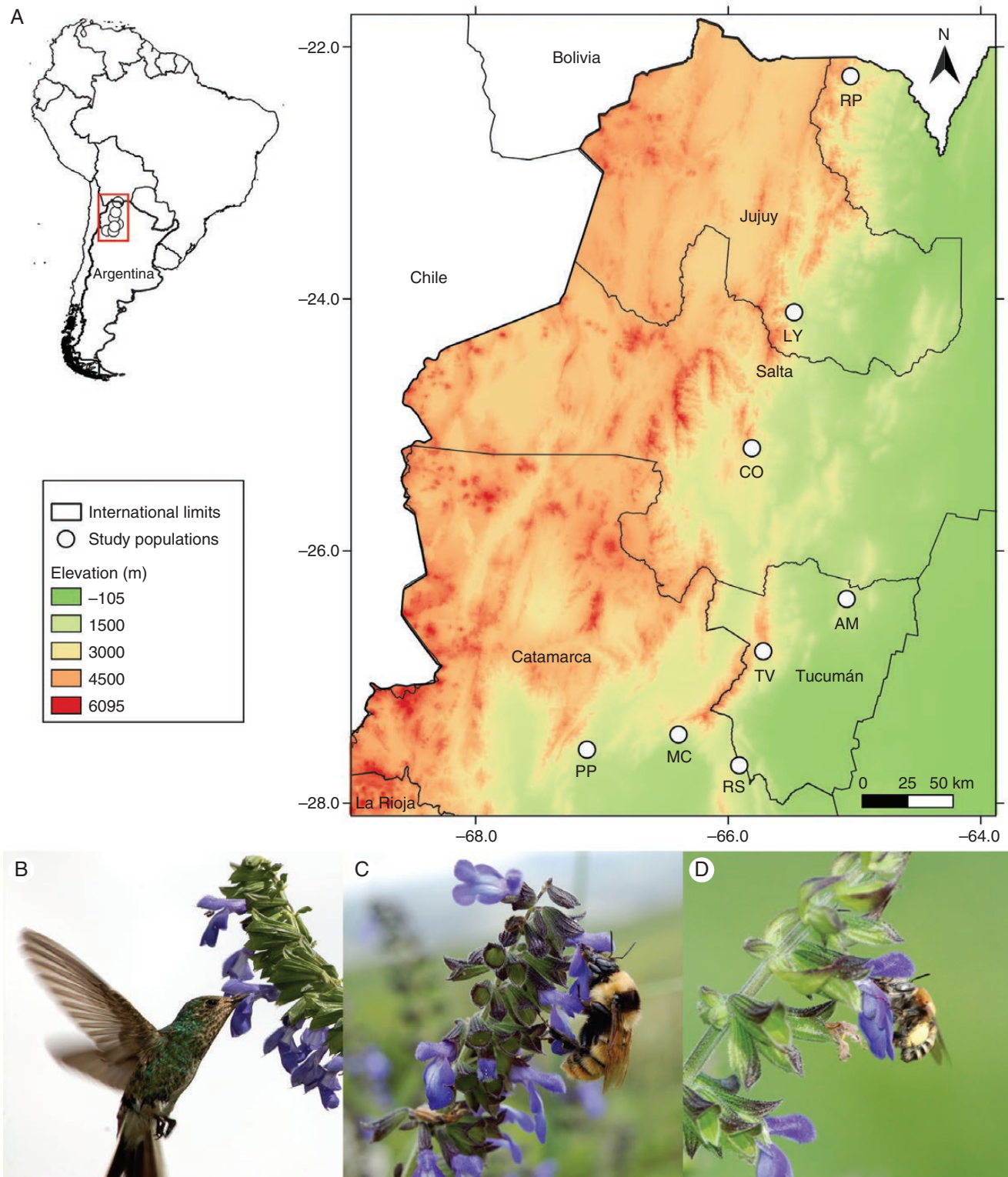


FIG. 1. Studied populations and main *Salvia stachydifolia* pollinators. (A) *Salvia stachydifolia* populations: PP, Poza de Piedra; MC, Minas Capillitas; RS, Río Singuil; TV, Tafi del Valle; AM, Altos de Medina; CO, Cuesta del Obispo; RP, Rodeo Pampa, and LY, Lagunas de Yala. Pollinators of *S. stachydifolia*; (B) A female *Sappho sparaganura* in Poza de Piedra; (C) a queen *Bombus opifex* in Tafi del Valle; and (D) *Anthophora paranaensis* in Altos de Medina. GBIF occurrence numbers for these records are 2265781233, 1453367975 and 2005315231, respectively.

the resulting coordinates. We then performed a principal components analysis (PCA) on those Procrustes coordinates. We also estimated the centroid size as a measure of corolla size. Centroid size is the square root of the sum of the square distances of all the landmarks to their centre of gravity, the location of which is obtained by averaging the  $x$  and  $y$  coordinates of all landmarks. To test for differences in floral shape and size among populations in natural and controlled conditions, we performed linear mixed models with Gaussian error structure, as described above. We used centroid size and the first two principal components of floral shape as response variables, plant identity as a random effect and population as a fixed effect. We log-transformed the centroid size before analyses (Zelditch *et al.*, 2012).

#### Ecotype detection

To classify flowers from the eight natural populations, we used an unsupervised learning algorithm known as Gaussian mixture analysis. This algorithm detects morphological discontinuities without any a priori classification, and it can be applied to multivariate data (Baylac *et al.*, 2003; Rubini Pisano *et al.*, 2019). Gaussian mixture analysis is particularly well suited to unsupervised delineation of clusters of points. However, it has one restriction: within-group distributions must be multivariate normally distributed (Baylac *et al.*, 2003). We applied this analysis in the reduced space of the first two principal components from geometric morphometric data and the log-transformed centroid size. We selected the most probable number of groups and their distributional properties using maximum-likelihood estimations (Fraley and Raftery, 1998). Our goal was to identify groups or clusters, with each one characterized by a multivariate mean and a covariance matrix. This matrix determines the geometric features of the clusters, including shape, volume and orientation, which may vary from group to group. To select the best model, we fitted models with an increasing number of clusters (from one to nine), and differing parametrizations in cluster means, shapes, volumes and orientations. We used the Bayesian information criterion (BIC) for model selection. We performed this analysis using the *mclust* R package (Scrucca *et al.*, 2016).

#### Climatic and elevation preferences

We explored whether populations with different pollinator assemblages had different climatic and/or elevation preferences, over to the whole geographical range of *S. stachydifolia*. Including all available occurrences is crucial to characterize the climatic multivariate space of this species. To achieve this, we built a database with geographical coordinates of the eight studied populations and occurrence data of *S. stachydifolia* from GBIF.org (4 June 2020) GBIF Occurrence Download <https://doi.org/10.15468/dl.3y2x2y>, iNaturalist database, as well as herbarium records from Fundación Miguel Lillo (LIL), Herbario Nacional de Bolivia (LPB), Herbario del Sur de Bolivia (HSB), Instituto Darwinion (SI), Instituto de Botánica del Nordeste (CTES) and Museo Botánico Córdoba (CORD). In case a record lacked a precise coordinate, we geolocated it when the locality information was accurate enough to allow

determining the precise site. We then filtered the data to remove records outside of the species' natural distribution area and those with large precision errors (>5 km). We randomly thinned the presence records to a minimum distance of 1 km. This process resulted in 134 unique occurrence records of *S. stachydifolia*.

Next, we obtained 19 bioclimatic variables and elevation raster layers from the WorldClim 2.1 database (Fick and Hijmans, 2017), cloud cover from the EarthEnv data repository (Wilson and Jetz, 2016), and global aridity index and potential evapotranspiration (Trabucco and Zomer, 2019). All layers were download at a spatial resolution of 0.5' (~1 km). We retained 13 variables after examining them for cross-correlation and discarding highly correlated variables ( $r > 0.90$ ). These variables included annual mean temperature (Bio 1), mean temperature diurnal range (Bio 2), isothermality (Bio 3), temperature seasonality (Bio 4), temperature annual range (Bio 7), annual precipitation (Bio 12), precipitation seasonality (Bio 15), precipitation of wettest quarter (Bio 16), elevation, cloud cover intra-annual variation, cloud cover annual mean, aridity index and potential evapotranspiration. We then performed a PCA to summarize the information (function *prcomp* in R software). Although we had a small sample of populations where pollinator data were recorded, we explored if populations sharing pollinator assemblages occupied the same area in multivariate space.

## RESULTS

#### Floral visitors

The main pollinator guilds visiting *S. stachydifolia* were bees and hummingbirds. We observed three hummingbird species: the red-tailed comet *Sappho sparganura*, which was present in six of the eight populations (Fig. 1B); the slender-tailed woodstar *Microstilbon burmeisteri*, which was observed in Lagunas de Yala and Rfo Singuil populations; and the blue-tufted starthroat *Heliomaster furcifer*, which was present in the Pozo de Piedra population. Main bee pollinators were bumblebees (almost exclusively *Bombus opifex* queens and workers, Fig. 1C), along with solitary bees from the genera *Anthophora* (Fig. 1D) and *Thygater*. We also recorded other bees including *Eufrisea mariana*, *Centris tricolor*, *Xylocopa* sp. and *Tapinotaspis* sp. All bees were observed foraging for nectar, and we did not record any pollen-collecting behaviour. Additionally, flies, mostly from the family Bombyliidae, were also observed visiting and pollinating *S. stachydifolia* at a low frequency in seven of the eight sites. Detailed records of pollinators per site, year and observation times can be found in [Supplementary Data Table S2](#).

Two populations, Lagunas de Yala and Pozo de Piedra, were predominantly pollinated by hummingbirds (hummingbird visits comprised >90 %, Fig. 2). Flies were the second most important group of pollinators in these populations, whereas the presence of bees was minimal. The Altos de Media and Minas Capillitas populations were almost exclusively pollinated by bees, with bee visits exceeding 98 % (Fig. 2). However, *Bombus opifex* bumblebees dominated the visits in Minas Capillitas, whereas they were absent in Altos de Medina, where

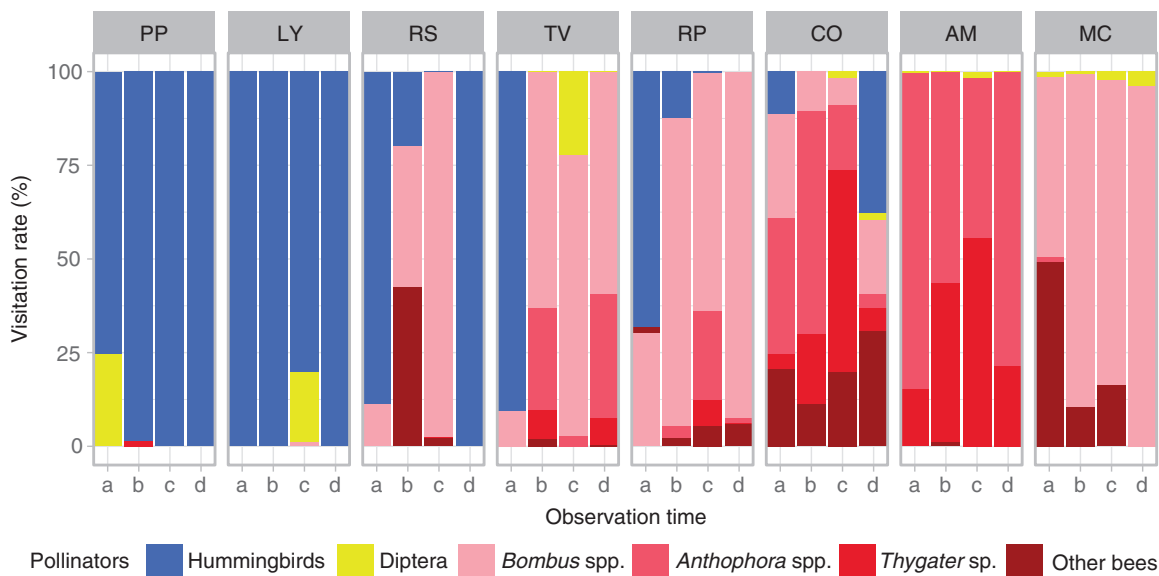


FIG. 2. Pollinator visits to *S. stachydifolia* at different observation times and in eight populations. Populations were ordered according to decreasing overall hummingbird visitation rate: PP, Pozo de Piedra; LY, Lagunas de Yala; RS, Río Singuil; TV, Tafi del Valle; RP, Rodeo Pampa; CO, Cuesta del Obispo; AM, Altos de Medina; and MC, Minas Capillitas. Observation times: a, morning (0800–0900 h); b, midday (1100–1200 h); c, afternoon (1500–1600 h); and d, evening (1800–1900 h). A complete record of visitation rates can be found in [Supplementary Data Table S2](#).

solitary bees were the main pollinators. Finally, four populations were pollinated by hummingbirds, bees and flies: Tafi del Valle, Río Singuil, Rodeo Pampa and Cuesta del Obispo (Fig. 2). In these populations most hummingbird visits were recorded in the morning and in the evening (Fig. 2 and [Supplementary Data Table S2](#)), while bees were the main pollinators during the rest of the day.

#### Nectar

Hummingbird-visited populations were characterized by high nectar volume but low concentration, both in natural and in common-garden conditions. The opposite pattern characterized bee- and mixed-pollinated populations, which had similar values in nectar volume and concentration (Fig. 3). There were significant differences in nectar volume among populations in both natural conditions [likelihood ratio test (LRT):  $\chi^2 = 312.85$ , d.f. = 7,  $P < 2.2 \times 10^{-16}$ , Fig. 3A upper panel] as well as in the common garden (LRT:  $\chi^2 = 143.24$ , d.f. = 7,  $P < 2.2 \times 10^{-16}$ , Fig. 3A lower panel). Also, there were significant differences in nectar concentration among populations in both natural (LRT:  $\chi^2 = 247.66$ , d.f. = 7,  $P < 2.2 \cdot 10^{-16}$ , Fig. 3B) and common garden conditions (LRT:  $\chi^2 = 48.89$ , d.f. = 7,  $P < 2.4 \times 10^{-8}$ , Fig. 3B).

#### Floral morphology

Hummingbird-pollinated populations displayed larger flowers with wide corolla opening and elongated tubes, while the opposite was true for bee- and mixed-pollinated populations. These differences are explained by variation in PC1, PC2 and centroid size (Fig. 4A). Variation in PC1 (48.72 % of the total variance) was associated with changes in corolla opening, i.e. the relative distance between the upper and lower lip.

Flowers with positive scores in PC1 were more closed, while those with negative scores were more open. Variation in PC2 (22.24 % of the total variance) corresponded to differences in the elongation of the corolla tube. Flowers with positive scores in PC2 had proportionally longer floral tubes, while those with negative scores had shorter tubes. Further PCs each accounted for <6.5 % of the total variance.

We found differences in PC1 among populations in both natural (LRT:  $\chi^2 = 176.48$ , d.f. = 7,  $P < 2.2 \times 10^{-16}$ ) and common garden conditions (LRT:  $\chi^2 = 20.16$ , d.f. = 7,  $P = 0.005$ ). However, differences between hummingbird-pollinated populations and the bee- or mixed-pollinated populations were not so prominent in the common garden compared with field conditions (Fig. 4B). We also found significant differences in PC2 among populations (Fig. 4C) in both natural (LRT:  $\chi^2 = 232.36$ , d.f. = 7,  $P < 2.2 \times 10^{-16}$ ) and common garden conditions (LRT:  $\chi^2 = 24.36$ , d.f. = 7,  $P < 9.8 \times 10^{-4}$ ). Finally, we found significant differences in centroid size (Fig. 4D) among populations both in natural (LRT:  $\chi^2 = 560.47$ , d.f. = 7,  $P < 2.2 \times 10^{-16}$ ) and in common garden conditions (LRT:  $\chi^2 = 84.44$ , d.f. = 7,  $P < 1.70 \times 10^{-15}$ ).

#### Ecotypes

Based on the Gaussian mixture analysis, the two best models (BIC < 2) included two distinct groups ([Supplementary Data Fig. S2](#)). After analysing the clusters from the two-group models, we found that all observations from bee- and mixed-pollinated populations belonged to cluster 1 (Table S3). Likewise, all the observations from the hummingbird-pollinated populations, except for four records from two individuals, belonged to cluster 2. By considering both the automatic cluster identification and population identity we concluded that there are two ecotypes, with an error in classification as low as 0.41 % (four in 960



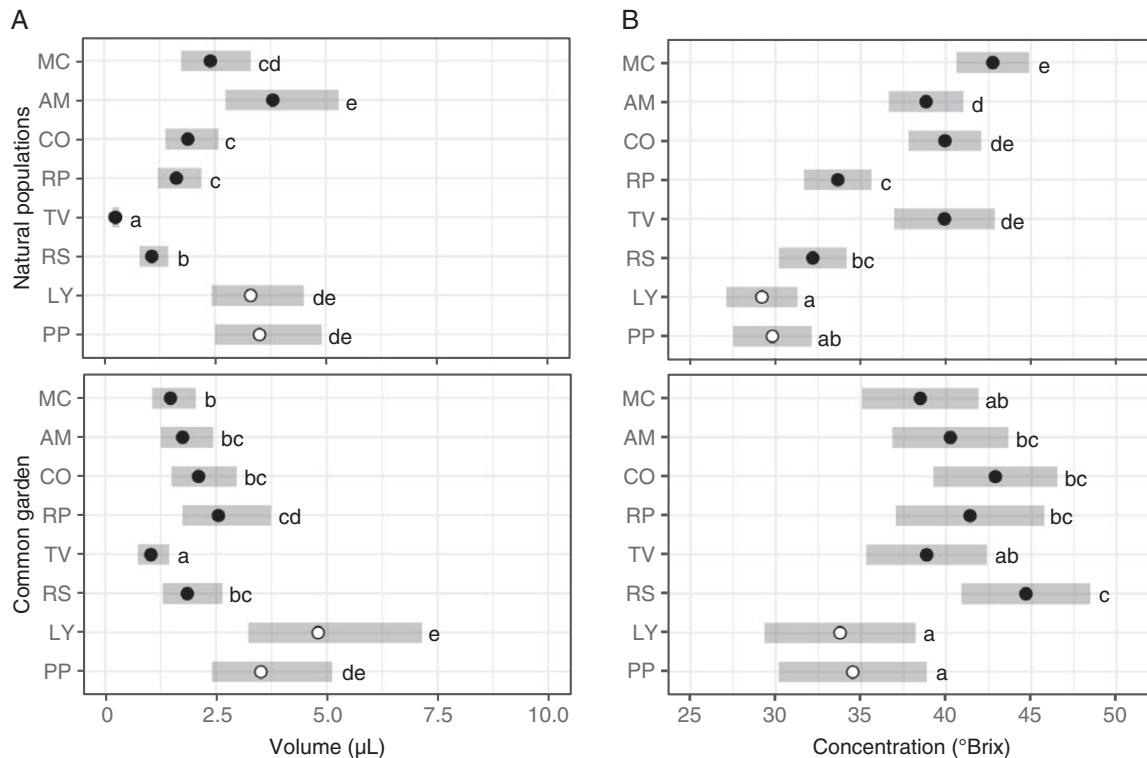


FIG. 3. Nectar traits in *Salvia stachydifolia* populations. (A) Nectar volume. (B) Nectar concentration. Results from natural populations (upper panels) and common garden (lower panels) are indicated. Different letters indicate significant differences in pairwise post-hoc Tukey comparisons with Bonferroni correction. Key to populations: PP, Pozo de Piedra; LY, Lagunas de Yala; RS, Río Singuil; TV, Tafi del Valle; RP, Rodeo Pampa; CO, Cuesta del Obispo; AM, Altos de Medina; and MC, Minas Capillitas. Open circles correspond to hummingbird-pollinated populations and closed circles to bee- and mixed-pollinated populations.

observations). In comparison with the second cluster, the first cluster consists of flowers with more positive scores in PC1 (i.e. more closed corollas), with negative scores in PC2 (less elongated corolla tubes) and of smaller size. Clusters showed multivariate normality, according to visual inspection.

#### Climatic and elevation preferences

The first three principal components represented 85 % of the total variation. PC1 explained 43 % of the total variation and was positively associated with mean temperature diurnal range, precipitation seasonality and elevation, while it was negatively associated with annual mean temperature, annual precipitation, precipitation of wettest quarter and the aridity index. PC2 explained 32 % of the total variation and it was positively associated with isothermality and cloud cover intra-annual variation, while it was negatively associated with temperature seasonality, temperature annual range and potential evapotranspiration. Finally, PC3 comprised 10 % of the total variation and was positively associated annual mean temperature and negatively associated with cloud cover annual mean (Supplementary Data Table S4).

The two hummingbird-pollinated populations did not occur in the same area of multivariate space. While the Lagunas de Yala population was close to the average climatic and elevation preferences of *S. stachydifolia*, the Pozo de Piedra population occurred in a marginal sector of multivariate space, characterized by low cloudiness, low precipitation, and comparatively

higher evapotranspiration and aridity (Fig. 5). The bee- and mixed-pollinated populations were scattered along PC1 and PC2 axes and close to PC3 mean (Fig. 5; Supplementary Data Fig. S3). When examining elevation, the median of the 132 occurrence records was 2265 m a.s.l. (range 384–4411 m a.s.l.). The studied hummingbird-pollinated populations have an elevation of 1859 m a.s.l. (Pozo de Piedra) and 2128 m a.s.l. (Lagunas de Yala), while bee- and mixed-pollinated populations range from 1476 to 3056 m a.s.l.

#### DISCUSSION

As expected under a local adaptation scenario, we found that differences in the identity of the principal pollinators were associated with variation among populations in floral shape, size and nectar traits. These differences persisted in the common garden, indicating that they had a genetic basis and were not due to phenotypic plasticity. *Salvia stachydifolia* is pollinated by hummingbirds, bees and flies, confirming previous observations (Barrionuevo et al., 2021). We did not find a continuum in visitation rates from hummingbird pollination to bee pollination. Instead, our results showed two populations that were almost exclusively pollinated by hummingbirds, while the other six populations had varying hummingbird visits ranging from 0 to 30 %. These differences corresponded to two floral ecotypes, which can be distinguished based on flower shape and size. The two populations from the hummingbird-pollinated ecotype are located in different mountain ranges, occupy different areas in

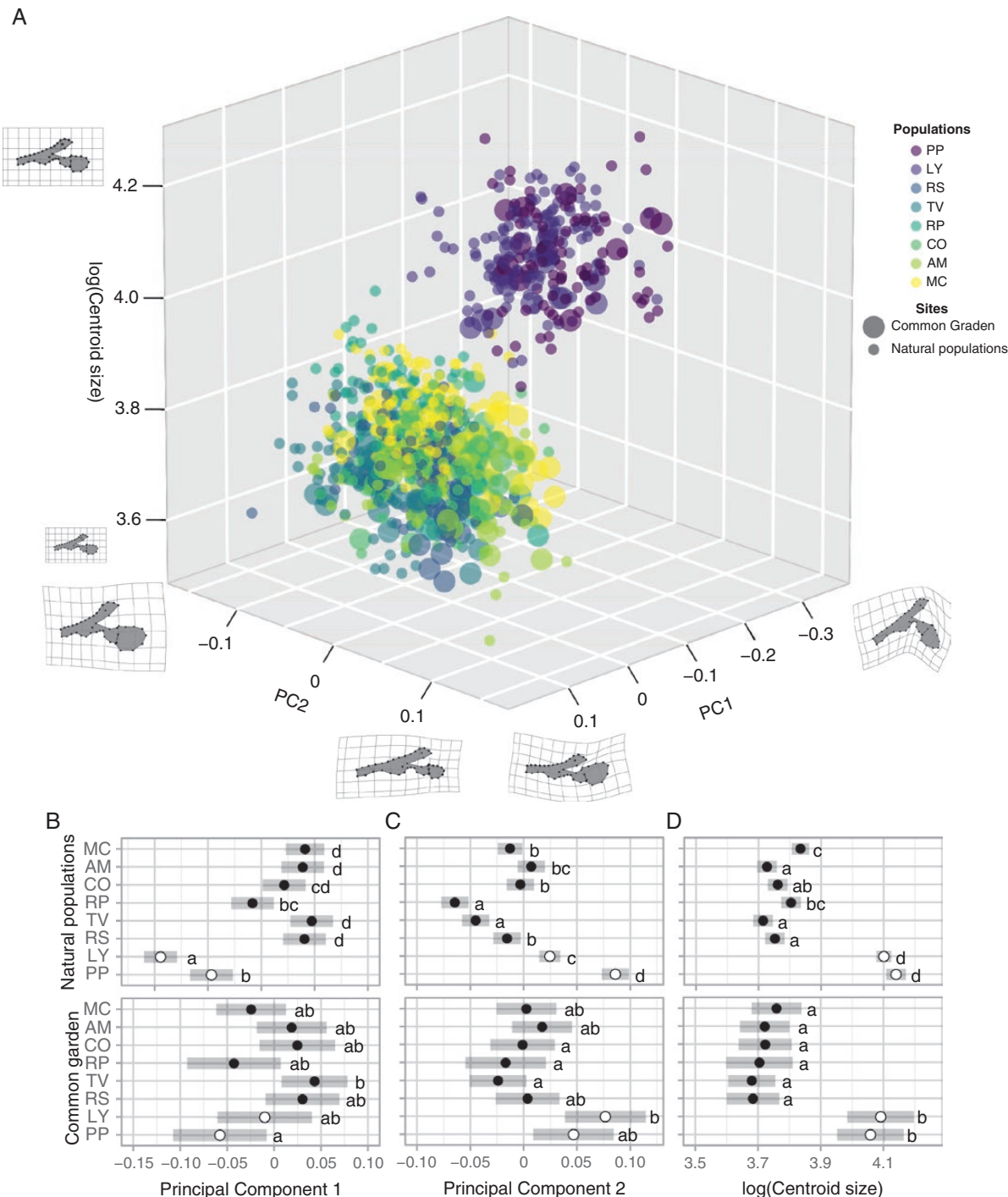


FIG. 4. Variation in flower shape and size in *Salvia stachydifolia*. (A) Flower shape and size (estimated as the first two principal components of Procrustes coordinates and logarithm of centroid size, respectively) variation in natural populations and the common garden. Maximum and minimum values from principal component 1, principal component 2 and corolla size are indicated with schematic flower shapes. Differences in *S. stachydifolia* populations in (B) principal component 1, (C) principal component 2 and (D) flower size, estimated as the logarithm of centroid size. Results from natural populations (upper panels) and the common garden (lower panels) are indicated. Different letters indicate significant differences in pairwise post-hoc Tukey comparisons with Bonferroni correction. Key to populations: PP, Pozo de Piedra; LY, Lagunas de Yala; RS, Río Singuil; TV, Tafi del Valle; RP, Rodeo Pampa; CO, Cuesta del Obispo; AM, Altos de Medina; and MC, Minas Capillitas. Open circles correspond to hummingbird-pollinated populations and closed circles to bee- and mixed-pollinated populations.

multivariate climate space and are separated a straight line distance of 420 km in, suggesting that gene flow between them is not currently occurring.

Variation in flower size among populations of *S. stachydifolia* has been noted in the taxonomic treatment for this species (Wood, 2007; O'Leary and Moroni, 2016). Here, we found



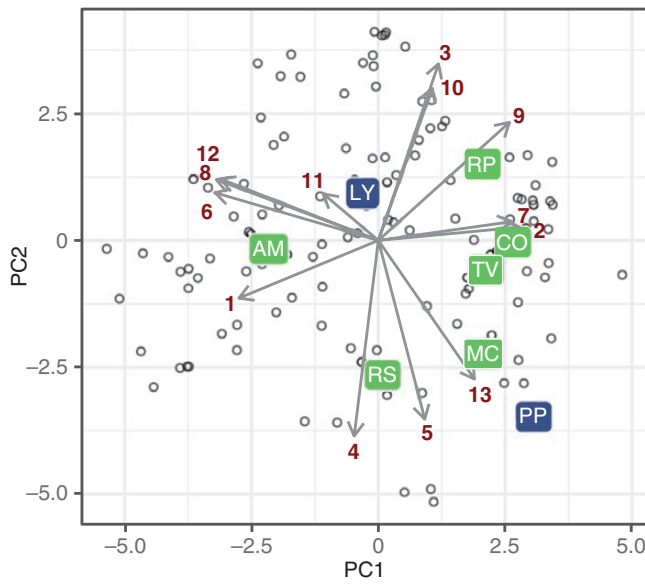


FIG. 5. Biplot of the principal component analysis showing the first two PCs. Loadings of variables (vectors) are indicated with arrows. Numbers indicate climatic and elevation variables: 1, annual mean temperature; 2, mean temperature diurnal range; 3, isothermality; 4, temperature seasonality; 5, temperature annual range; 6, annual precipitation; 7, precipitation seasonality; 8, precipitation of wettest quarter; 9, elevation; 10, cloud cover intra-annual variation; 11, cloud cover annual mean; 12, aridity index; and 13, potential evapotranspiration. Open circles indicate *S. stachydifolia* records from databases. Boxes correspond to the studied populations: bee- and mixed-pollinated populations are in green, while hummingbird-pollinated populations are in blue. Key to populations: PP, Pozo de Piedra; LY, Lagunas de Yala; RS, Río Singuil; TV, Taff del Valle; RP, Rodeo Pampa; CO, Cuesta del Obispo; AM, Altos de Medina; and MC, Minas Capillitas.

two ecotypes using geometric morphometrics and an unsupervised learning algorithm. The main traits that support these ecotype differences are floral size and PC2, which is related to elongation of the corolla tube. Variation in PC1, related to corolla opening, was more evident in natural populations than in common garden conditions, indicating that it may be a more plastic trait. Corolla tube length is an important determinant of pollinator preferences, and its evolutionary lability suggests that the developmental changes underlying length differences could be relatively simple, as seen in other plant species (e.g. Gurung *et al.*, 2021). It is worth mentioning that we did not analyse other aspects of floral and plant phenotype that might influence pollinator visits and behaviour, such as colour, plant height or stigma position, which will deserve future attention.

The variations in flower size and shape found in *S. stachydifolia* align with the pattern identified in macroevolutionary studies, despite being less pronounced. In a study comparing 18 *Salvia* species, Benitez-Vieyra *et al.* (2019) found that the main differences between bee- and hummingbird-pollinated *Salvia* species involved flower size and shape. Likewise, a broader study by Kriebel *et al.* (2020) found that corolla, anther connective and style shape were associated with different pollinators in *Salvia*. The differences in nectar traits among *S. stachydifolia* populations were also consistent with these trends (Benitez-Vieyra *et al.*, 2014; Saravia *et al.*, 2023), with hummingbird-pollinated populations displaying low concentration and comparatively high nectar volume. These differences are notably in the

common garden while some variation in nectar traits under natural conditions may be due to local differences in temperature and humidity.

Other cases of mixed pollination systems in *Salvia* may offer opportunities to study ongoing shifts in pollinators. For instance, *S. virgata* and *S. verticillata* are pollinated by both bees and flies (Celep *et al.*, 2014), while *S. rhombifolia* (Cairampoma *et al.*, 2020), *S. purpurea* and *S. mexicana* (Wester and Claßen-Bockhoff, 2011; Benitez-Vieyra *et al.*, 2014) are pollinated by both bees and hummingbirds. Other Andean *Salvia* species may also exhibit mixed pollination and population divergence in floral traits (Saravia *et al.*, 2023). In addition, many *Salvia* species do not fit in either the bee or hummingbird pollination syndrome, according to Wester and Claßen-Bockhoff's (2011) comprehensive classification, suggesting they would be mixed-pollinated. More empirical pollinator observations are needed to characterize pollination systems in *Salvia*, as the presence of secondary pollinators and inter-population variation may be overlooked (e.g. Cairampoma *et al.*, 2020; Saravia *et al.*, 2023). This limitation may be common, particularly affecting macroevolutionary studies (van der Niet, 2021).

According to the last available phylogenies of *Salvia* (Fragoso-Martínez *et al.*, 2018; Kriebel *et al.*, 2019), *S. stachydifolia* belongs to the 'South American Clade'. However, phylogenetic relationships remain uncertain within this clade. The common ancestor between *S. stachydifolia* and its closest relative, the bee-pollinated *S. sophrona*, was hummingbird-pollinated according to Kriebel *et al.* (2019), or bee-pollinated according to Sazatornil *et al.* (2023), who applied a different reconstruction method. Despite the disagreements regarding the ancestral reconstruction, all studies agree that pollination systems are prone to change and bird pollination is not a dead end (Fragoso-Martínez *et al.*, 2018; Kriebel *et al.*, 2019; Sazatornil *et al.*, 2023). We suggest that bee pollination is the most likely ancestral state of *S. stachydifolia* due to the prevalence of bee- or mixed-pollinated populations. However, until genealogical relationships among populations are resolved, this question will remain unresolved.

Previous studies of pollinator-driven local adaptation have focused primarily on generalist plant species, such as *Erysimum mediohispanicum* (Gómez *et al.*, 2009; Gómez and Perfectti 2010). In this species, spatial variation in flower traits was associated with plant–pollinator interactions and not with the genetic distances between populations. Likewise, Parker *et al.* (2018) found that divergence in *Claytonia virginica* flower traits was related to two different pollinator climates, dominated by pollen-specialist bees in northern populations and by generalist bee-flies in the southern ones. To our knowledge, the present study is the first to address local adaptation to hummingbird or bee pollination within a single species with systematic pollinator records. *Euphorbia tithymaloides* (Euphorbiaceae) may represent a similar case, displaying populations either pollinated by hummingbirds or with generalized pollination associated with insularity (Cacho *et al.*, 2019; Cacho and José-Zacatula, 2020). Adaptation to bees and hummingbirds has been additionally recorded in closely related species such as *Mimulus lewisii* and *M. cardinalis* (Schemske and Bradshaw 1999), *Clarkia concinna* and *C. breweri* (Miller *et al.*, 2014), and other species pairs in the genera *Penstemon*, *Ipomoea*, *Costus* and *Silene* (reviewed in Thomson and Wilson, 2008). We are

aware that more multi-year pollinator observations are needed in the studied *S. stachydifolia* populations to characterize pollinator assemblages. Nonetheless, the absence of bees visiting *S. stachydifolia* in the hummingbird-pollinated populations, even when they were present in the pollinator community, suggests some *S. stachydifolia* populations may have evolved some ‘anti-bee’ adaptations, such as larger flower size and low-concentration nectar, which have been previously observed in other plant species (Castellanos *et al.*, 2004; Gegeer *et al.*, 2017). However, this hypothesis remains to be tested.

Many hypotheses have been proposed to explain shifts from bee to hummingbird pollination. First, hummingbirds may be more efficient at pollen delivery because they neither feed on pollen nor display grooming behaviour, whereas bees groom themselves and have specialized pollen-carrying structures, resulting in pollen wastage. We did not find support for this hypothesis, because we did not record any interaction involving pollen collection by bees. In addition, the lever mechanism of *Salvia* and nototribic pollen placement makes grooming more difficult. The second hypothesis, proposed by Thomson and Wilson (2008), suggested that extrinsic environmental factors may trigger a change in the identity of the most efficient pollinator, resulting in a different stabilizing selection regime. Altitude is the most important environmental factor in mountain habitats, impacting on pollinators’ effectiveness (Cruden, 1972), and recent studies in Andean plant clades have shown that pollinator shifts coincide with recent phases of mountain uplift (Lagomarsino *et al.*, 2017; Serrano-Serrano *et al.*, 2017; Dellinger *et al.*, 2021). In addition, Dellinger *et al.* (2021) demonstrated that vertebrates were more efficient pollinators than bees under the colder and rainier conditions of tropical mountains. We observed hummingbirds dominating flower visits during the cold hours of the day in mixed-pollinated populations, suggesting that they may be more efficient pollinators than bees in colder conditions. However, contrary to these expectations, the two hummingbird-pollinated populations do not share common climatic conditions and one of them, Pozo de Piedra, is the most arid of the studied populations with a comparatively low altitude, even though it is located above 1000 m a.s.l. and cannot be characterized as a ‘lowland’ population (Dellinger *et al.*, 2023).

Here we propose a third, non-exclusive hypothesis, that may help to understand rapid pollinator shifts. As widely acknowledged, hummingbirds may behave as opportunistic foragers (Leimberger *et al.*, 2022) depending on local floral resources, i.e. hummingbirds often visit non-ornithophilous flowers, behaving as generalist pollinators. Profuse *S. stachydifolia* populations may constitute a favourable habitat for hummingbirds such as *Sappho sparganura* to establish their feeding territory and nests (Contino, 1975). In such conditions, a positive feedback may arise because selection for ‘pro-bird’ and ‘anti-bee’ floral traits in the plant populations may favour maintenance of the hummingbird population in the long term. Such eco-evolutionary dynamics can lead to the rapid evolution of local adaptation and even speciation (Lewontin, 2001; Hendry, 2016). The trigger for these dynamics depends largely on chance, and thus our hypothesis can better fit patterns of divergence as a consequence of historical isolation on mountaintops. *Salvia stachydifolia* occurs in a narrow area of vegetation, in isolated patches across different mountain ranges. For instance, Pozo de Piedra, Minas

Capillitas and Río Singuil are populations separated by narrow dry valleys, with a total straight line distance between them of only 127 km (Fig. 1A), and they strikingly differ in their pollinator fauna. Here we suggest that relatively recent geological and climatic changes, such as the Pleistocene oscillations in the Southern Andes, may have left a strong signature in the structuring of population divergence (e.g. Sosa-Pivatto *et al.*, 2017).

Future genetic studies will allow us to disentangle the history of isolation along the distribution range of *S. stachydifolia*. We suggest that differences among *S. stachydifolia* populations in floral shape, size and nectar traits point to a scenario of incipient speciation, as a result of reproductive and geographical isolation. This study provides the first approximation to understanding the processes underlying among-population differentiation and, possibly, reproductive isolation in an Andean species with different pollinator guilds. Population genetic analyses will be needed to confirm the local adaptation scenario proposed (e.g. Leal *et al.*, 2021). In that sense, although pollinator-driven divergent selection and isolation seems to be the main factor driving the observed phenotypic pattern, both genetic drift and gene flow may also contribute, at least in part, to the among-population phenotypic variation (e.g. Baranzelli *et al.*, 2014). The comparison of neutral and adaptive genomic variation among populations would allow valuable insights into the evolutionary processes driving phenotypic structure in natural populations. In the future, we therefore expect to compare phenotypic and genetic patterns among populations to confirm the mechanisms responsible for population differentiation in *S. stachydifolia*.

#### SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following.

**Table S1.** Geographical location of the study sites and the common garden.

**Table S2.** Pollinator visitation rates to *Salvia stachydifolia*.

**Table S3.** Best models ( $\Delta\text{BIC} < 2$ ) according to Gaussian mixture analysis.

**Table S4.** Principal component loadings of bioclimatic variables.

**Figure S1.** Positions of landmarks and curves that describe the corolla shape of *S. stachydifolia*.

**Figure S2.** Floral morphology in *Salvia stachydifolia* populations.

**Figure S3.** Biplots of the principal component analysis showing the first three PCs.

#### AUTHOR CONTRIBUTIONS

J.V.I. and S.B.V. designed the study. J.V.I. performed measurements and statistical analysis. S.C. and M.B. collected and analysed geographical and bioclimatic data. All authors contributed to field observations and contributed to the final manuscript.

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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