

**Floral biology of *Salvia stachydifolia*, a species visited by bees and birds: connecting sexual phases, nectar dynamics and breeding system to visitors' behaviour**

Camila N. Barrionuevo, Santiago Benitez-Vieyra & Federico Sazatornil\*

Instituto Multidisciplinario de Biología Vegetal (Universidad Nacional de Córdoba – CONICET), CC 495 (X5000ZAA), Córdoba, Argentina.

\* Corresponding author: [federicosaza@gmail.com](mailto:federicosaza@gmail.com)

This article has been accepted For publication And undergone full peer review but has Not been through the copyediting, typesetting, pagination And proofreading process, which may lead to differences between this

Version And the Version of Record. Please cite this article as doi: 10.1093/jpe/rtab012.

© The Author(s) 2021. Published by Oxford University Press on behalf of the Institute of Botany, Chinese Academy of Sciences and the Botanical Society of China. All rights reserved. For permissions, please email: [journals.permissions@oup.com](mailto:journals.permissions@oup.com)

## ABSTRACT

### *Aims*

Adaptive convergence in floral phenotype among plants sharing a pollinator guild has been acknowledged in the concept of pollination syndrome. However, many plants display traits associated with a given syndrome, but are visited by multiple pollinators. This situation may indicate the beginning of a pollinator shift or may result in a stable situation with adaptations to different pollinators. In *Salvia stachydifolia*, a previous study suggested that flower shape is optimized to maximize the contribution to pollination of bees and hummingbirds. Here, we studied three additional aspects of its floral biology: sexual phases, nectar dynamics and breeding system, and examined their connection with pollinators' behavior to explore the presence of adaptations to bee and/or hummingbird pollination.

### *Methods*

Using a greenhouse population, we applied five pollination treatments to characterize breeding system. To determine sexual phases, we recorded flower opening, anther dehiscence, corolla fall and stigma receptivity. Additionally, we characterized nectar volume and concentration dynamics along the day. Finally, to determine pollinator assemblage and visitation patterns, we performed field observations and recorded pollinators' behavior.

### *Important findings*

*Salvia stachydifolia* was partially protandrous and self-compatible, but open-pollinated plants attained the highest reproductive success, suggesting that reproduction is mainly dependent on pollinator activity. *Bombus opifex* bumblebees were the most frequent visitors, but *Sappho sparganura* hummingbirds dominated visits early in the morning and at dusk. Nectar was typical of bumblebee-pollination. We suggest that the bee-hummingbird mixed visitation constitutes an unstable evolutionary situation, making *S. stachydifolia* an ideal system to understand the ecological circumstances in which pollination shifts occur.

KEY WORDS: *Salvia*; sexual phases; nectar dynamics; breeding system; pollinator behavior.

Accepted Manuscript

## INTRODUCTION

The adaptive convergence in floral traits among plants that share the same functional group of pollinators, is a phenomenon acknowledged in the well-known concept of pollination syndromes (van der Pijl 1961; Fenster *et al.* 2004; Dellinger 2020). The fundamental assumption underlying this concept is that flowers adapt to their single most efficient functional pollinator group (Stebbins 1970; Dellinger 2020). However, evolutionary specialization into pollination syndromes does not preclude the existence of secondary, potentially less efficient pollinators (Stebbins 1970; Fenster *et al.* 2004; Rosas-Guerrero *et al.* 2014; Dellinger 2020). Actually, many plants display traits commonly associated with a given pollination syndrome, but they are visited by multiple pollinator guilds. Beyond the presence of generalist plants, accessible to a wide diversity of pollinators, pollination by two pollinator guilds could result in mixed (Salas-Arcos *et al.* 2017) or bimodal (Manning and Goldblatt 2005; Dellinger *et al.* 2019) stable pollination systems. Floral adaptation to several pollinators can evolve if it results in an overall fitness gain (Aigner 2001, 2006) and if pollinator-mediated adaptive trade-offs are minimal (Thomson 2003; Castellanos *et al.* 2004; Muchhala 2007; Strelin *et al.* 2017; Dellinger *et al.* 2019).

Pollinator fauna may vary in space and time due to random fluctuation in visitors abundances, the occasional presences of the alternative floral resources or transient changes on weather conditions. However, the coexistence of two pollinator guilds can also indicate the presence of an evolutionary unstable situation as the beginning of a shift towards new pollinators (Thomson and Wilson 2008; Manning and Goldblatt 2005; Salas-Arcos *et al.* 2017). Mixed pollination has been suggested as a first step in the transition from bee- to hummingbird-pollination (Thomson and Wilson 2008; Salas-Arcos *et al.* 2017), one of the most common cases of pollinator shifts in New World plant clades (Grant and Grant 1968; Thomson and Wilson 2008; Abrahamczyk and Renner 2015). The ecological context plays a major role in the increase of hummingbirds' visits. For instance, colonizing plants may face new habitats where bees are scarce, or hummingbirds may be active when bees are not (due to cold weather or fog during part of the day, Cruden 1972; Salas-Arcos *et al.* 2017; Cairapoma *et al.* 2020). If the new ecological conditions become persistent, natural selection should favor flower traits that

promote visits by hummingbirds and disfavor visits by bees (Castellanos *et al.* 2004; Thomson and Wilson 2008; Salas-Arcos *et al.* 2019; Wester *et al.* 2020).

The genus *Salvia* involves about 1000 species, from which about 600 belong to the American subgenus *Calosphace*. According to Wester and Claßen-Bockhoff (2011) 31% of *Calosphace* can be classified as hummingbird-pollinated, 59% are likely pollinated by bees, and about 10% are not clearly assignable to either pollinator syndrome. Phylogenetic comparative analyses suggested that shifts from bee- to hummingbird-pollination, as well as reversals, have occurred many times in *Calosphace* (Fragoso-Martínez *et al.* 2018; Kriebel *et al.* 2019). Recently, Wester *et al.* (2020) proved that floral morphology excludes bees in hummingbird-pollinated *Salvia*, while colour patterns might play a role as a floral filter against bees in some species. Both hummingbird and bee-pollinated species were characterized as auto-compatible and predominantly outbreeders, but showed different levels of self-pollination (Haque and Goshal 1981; Cuevas-García *et al.* 2013; Rosas-Guerrero *et al.* 2017; Cuevas *et al.* 2018). Haque and Goshal (1981) pointed out that the small and blue flowered species show higher levels of self-pollination than the large and red flowered species. Self-compatibility has been proposed as a preadaptation for certain types of pollinator transitions, like from bee to hummingbird pollination, or for the evolution of pollinator specialization (Wessinger and Kelly 2018), suggesting that breeding system can play a role favouring pollinator shifts in *Salvia*.

*Salvia stachydifolia* Benth., a species from the Andean region of Argentina and Bolivia, fits many characteristics of a bee-pollinated species: a bilabiate blue corolla, with a lower lip providing a landing platform and a short corolla tube (Wester and Claßen-Bockhoff 2011; Benitez-Vieyra *et al.* 2019). Bees were recorded as flower visitors by Wester and Claßen-Bockhoff (2011). Later, Strelin *et al.* (2017) reported both bees and hummingbirds visiting their flowers and that hummingbirds transported pollen on their beaks in an Argentinian population. These authors also suggested that pollination by bees and hummingbirds in *S. stachydifolia* involved the optimization of flower shape configuration that maximize the contribution of each type of pollinator (Strelin *et al.* 2017). Recent studies suggest that mixed pollination may not be uncommon in other *Salvia* species, despite the presence of floral traits associated with bee pollination (Cairampoma *et al.* 2020).

Optimization of flower traits other than flower shape in bee-hummingbird mixed pollinated species do not necessarily lead to an intermediate state. For instances, nectar concentration typical of bee-pollinated species do not exclude hummingbird visits, but the low nectar concentration of hummingbird-pollinated flowers has been suggested as an anti-bee adaptation (Castellanos *et al.* 2003; Thomson and Wilson 2008; Salas-Arcos *et al.* 2019; Wester *et al.* 2020). On the other hand, nectar production patterns have been linked to differences in pollinators foraging activities (e.g. Salas-Arcos *et al.* 2017) and we can expect the same pattern if *S. stachydifolia* is adapted to both pollinator guilds.

Dichogamy (temporal separation of sexual functions) has been associated with insect pollination (Willmer 2011). In particular, protandry (the early maturation of male reproductive elements) has been associated with bee pollination but it is uncommon in hummingbird pollinated species (Sargent and Otto 2004). This association can be explained as a consequence of the differences between bees and birds foraging behaviours while bees visiting vertical inflorescences tend to consistently start at the bottom and move upwards (Harder *et al.*, 2004), hummingbirds' movements are less stereotypical (Sargent and Otto 2004). Thus, in self-compatible species, bee-pollination could promote geitonogamy and inbreeding depression and, in absence of pollen limitation, these conditions may lead to the evolution of complete dichogamy (Rosas Gerrero *et al.* 2017). Contrarily, partial dichogamy is expected in an intermediate bee-hummingbird visited species, like *S. stachydifolia*.

In this paper we studied three aspects of the reproductive biology of *S. stachydifolia*: sexual phases, nectar dynamics and breeding system, and examined their connection with visitors' behaviour. If *S. stachydifolia* is adapted to both bee and hummingbird pollination, we expect that nectar production matches the peaks of visitors foraging activity. Additionally, because selection on dichogamy should be reduced in hummingbird or mixed-pollinated species, we expect *S. stachydifolia* to be partially dichogamous or adichogamous. As this premise depends on differences in pollinator behaviour and on the presence of self-compatibility, we additionally recorded the number of

consecutive flowers visited within a plant by the main visitors, and characterized *S. stachydifolia* breeding system.

## METHODS

### *Study system*

*Salvia stachydifolia* Benth. (Lamiaceae) is a perennial herb with tuberous rhizomes and erect stems, 1-2 m in height. It grows between 1500-3500 m a.s.l. in humid mountain grasslands of northern Argentina and central-southern Bolivia. Inflorescences are paniculated, with many blue flowers arranged in pseudoracemes up to 20 cm long. Flower architecture is bilabiate, the corolla length varies along their geographical distribution from 13 to 25 mm (O'Leary and Moroni 2016). It has a ~12 mm tube, a ~7 mm long pubescent upper lip and a ~ 12 mm long glabrous lower lip, often with white nectar guides (Wood, 2007; O'Leary and Moroni 2016). Gynoecium is formed by two fused carpels, each divided in two loculi. Androecium has two stamens with a lever mechanism that deposits pollen on the pollinator body. Nectar is secreted and accumulated at the base of the corolla tube.

We performed field observations of pollinator visits and behaviour at a population located near Tafi del Valle (Tucumán province, Argentina; 26°47'48'' S, 65°43'22'' W, 3021,59 m a.s.l.). The population occurs in a humid slope alongside a mountain road and it involves about 60 plants. Other flowering species at the site were *Salpicroa tristis*, *Caiophora clavata*, *Solanum* spp., *Brassica rapa*, *Achantolippia* sp., *Stevia* sp. and dwarf *Maytenus boaria* individuals. To study breeding system, sexual phases and nectar dynamics we used potted plants (except in the case of the open pollination treatment), obtained from seeds from 60 parental plants of Tafi del Valle population in 2015, and grown in a greenhouse at the Instituto Multidisciplinario de Biología Vegetal (Córdoba, Argentina).

### *Sexual phases*

We recorded flower opening, anther dehiscence (beginning of the male phase) and corolla fall in 70 flowers from 30 individuals. All flowers opened with unfolded stigma branches, thus the beginning of the female phase was determined by testing receptivity in five to eight stigmas, each from a different plant, with hydrogen peroxide (5% v/v, Dafni *et al.* 2005) at different hours (from 3:00 h to 18:00 h). High peroxidase activity has been found to be correlated with stigmatic receptivity (Knox *et al.* 1986). However, it is worth mentioning that this technique can give false positive results, particularly from old stigmas (Dafni *et al.* 2005). We classified stigmas in a qualitative scale as non-receptive, intermediate or receptive, according to increasing bubbling intensity.

### *Nectar dynamics*

We sampled first-day flowers each hour between 6:00 and 21:00 h (n = 80). The previous day buds were bagged with fine-mesh bags to allow nectar accumulation and to avoid animal visits. In each sample, we harvested five flowers from different plants and we measured nectar volume ( $\mu\text{l}$ ) using 5ml calibrated micro caps (BLAUBRAND®) and then we recorded nectar concentration ( $\mu\text{g}/\mu\text{l}$  and Brix) using hand refractometers (0-32 and 28-52 brix, Atago®). A total of 80 flowers from 21 different plants were included in analyses. We examined patterns of nectar production through generalized additive mixed models (GAMMs). Additive models extend the standard linear model by allowing non-linear functions of each of the variables, providing a more accurate description of how nectar volume and concentration varied over time. In addition, we included plant identity as a random effect to account for repeated measures in the same plant. For this, we used the *gam4* function of *gam4* package (Wood and Scheipl 2017) in R software (R Core Team 2019). Smoothing parameters were obtained by minimizing the generalized cross-validations scores (Wood, 2008), and Bayesian standard errors were obtained according to Wood (2006).



### *Breeding system*

We performed five treatments: a) *open pollination*, non-manipulated flowers were exposed to pollinators at the study site; b) *autonomous self-pollination*, buds were bagged throughout their anthesis period; (3) *hand self-pollination*, bagged buds were emasculated and pollinated by hand with pollen from other flowers of the same plant; (4) *hand outcross-pollination*, bagged buds were emasculated and pollinated by hand with pollen of recently opened flowers of other individuals; (5) *apomixis*, bagged buds were emasculated. In all cases, fruits were collected 10 days after pollination. In each treatment, 30 to 36 flowers were used, from 29 different plants (at least 7 different plants per treatment). In the case of the apomixis treatment, stigmas were collected after flower fall, mounted on slides, stained with phloxine and methylene green (Dafni *et al.* 2005) and inspected under the microscope in order to rule out self-pollination.

We estimated reproductive success as the proportion of flowers that set fruits and as the proportion of ovules that set seed per flower (as in all *Salvia*, flowers bear four ovules). To test for significant differences among treatments, we applied generalized linear mixed models (GLMM) with binomial error structure. Plant identity was incorporated as a random effect and treatment as a fixed effect. Post hoc (Tukey) tests between pairs of marginal means were conducted using the *emmeans* R package (Lenth 2018). In addition we estimated the self-incompatibility index (ISI) dividing the average number of seeds produced under hand self-pollination over the average number of seeds under hand cross-pollination. According to Ruiz Zapata and Arroyo (1978), self-compatible species score  $ISI = 1$ , incompletely compatible species take ISI values between 0.2 and 1 and values of  $ISI < 0.2$  correspond to self-incompatible species. We also estimated the automatic self-pollination index (IAS) dividing the average number of seeds under autonomous self-pollination with the average number of seeds under hand self-pollination. In this case, fully autogamous plants score  $IAS = 1$ , while partially autogamous plants score  $IAS < 1$  (Ruiz Zapata & Arroyo, 1978).

## *Flower visitors and behaviour*

We recorded visitation rate during 58 periods of 30 min (21 in 2017 and 35 in the 2018 flowering season), distributed between 7:00 and 21:00 h. We only recorded visitors that touched the anthers or the stigma, thus only including those visitors that can be considered pollinators. We classified visitors according to three main pollinator guilds (hummingbirds, bees and flies) and identified them to species level when possible. As we observed remarkable differences in size and behaviour, we separately recorded queen and worker bumblebees. We examined the daily patterns of pollinator activity. Data from different flowering seasons were pooled, as the main four pollinators were the same in both years.

We examined pollinator behaviour, considering the number of consecutive flower visits inside a plant. We tested for differences in pollinator behaviour between the two main functional pollinator groups (hummingbirds vs. bees) and between the four main pollinators. Both tests were performed using a generalized linear model with Poisson error structure and quasi likelihood estimation, as overdispersion was detected. Year was incorporated as a fixed variable.

## RESULTS

### *Sexual Phases*

Flowers of *Salvia stachydifolia* began to open at 3:00 h and they reached a peak of open flowers (all 70 flowers open at the same time) at 7:30 h. Flowers had a maximum lifespan of seven days (mean = 3.971; s.d. = 1.296; Fig. 1a). Even though some anthers were closed when a flower opens, this phase in flower life was extremely short, involving less than one hour. Thus, only a small portion of the flowers in the population displayed closed anthers during their first day of life, and at 9:00 h all flowers had their pollen exposed (Fig. 1a).

All flowers showed unfolded stigmatic branches when opening, but most stigmas were not receptive at 3:00 and 6:00 h, indicating that flowers were in male phase, with a small fraction

presumably in neutral phase (closed anthers and not-receptive stigmas). That situation changed around 9:00 h, with a majority of flowers displaying medium to high stigma receptivity and dehiscent anthers, being thus in the hermaphroditic phase (Fig. 1b). The proportion of flowers with fully receptive stigmas continued to increase, to a peak at 18:00 h of the first day. Receptivity continued high during the second and third days. Stigmas began to lose their receptivity at 18:00 h of the third day, indicating the beginning of flower senescence (Fig. 1b).

### *Nectar dynamics*

Average nectar volume was 1.81  $\mu\text{l}$  ( $\pm 1.03$  s.d.) while concentration was 44.68 Brix ( $\pm 8.91$  s.d.). Nectar concentration showed two peaks ( $F= 2.78$ ;  $p= 0.02$ ), around 8:00 h and 16:00 h, and a minimum at 12:00 h (Fig. 2a). In turn, nectar volume showed a steady increase along the measurement period, attaining its maximum at 21:00 h (Fig. 2b). It worth this result must be taken with caution, because we did not measure nectar volume and concentration along the complete lifespan of the flowers.

### *Breeding system*

There were significant differences among treatments in fruit set (likelihood ratio test,  $\chi^2 = 34.031$ ,  $P < 0.0001$ , Fig. 3a). The probability of setting fruits was above 0.5 in the open pollination, hand self-pollination and hand cross-pollination treatments, while it was below 0.3 in the apomixis and autonomous self-pollination treatments. A similar pattern was found when the average number of seeds was used as an estimate of reproductive success (likelihood ratio test,  $\chi^2 = 96.948$ ,  $P < 0.001$ , Fig. 3b). The proportion of ovules that set seeds was higher in the open pollination, hand self-pollination and hand cross-pollination treatments. The apomixis and autonomous self-pollination treatments produced less than 1 seed per fruit on average.

Plants of *S. stachydifolia* from the studied population were self-compatible (ISI = 0.816) and partially autogamous (IAA = 0.374). There was no evidence of pollen limitation, as the average

number of seeds and fruit set were similar between the open pollination and the hand cross-pollination treatments.

#### *Flower visitors and behaviour*

A total of 3181 interactions (flower visitors contacting anthers or stigma) were recorded during the observation periods across two flowering seasons. Main flower visitors belonged to three guilds (Table 1): bees (85% of the visits, mainly from the genus *Bombus* and *Anthophora*), *Sappho sparganura* hummingbirds (12 % of the visits) and dipterans (3%). Main visitor guilds (bees and hummingbirds) visiting *S. stachydifolia* flowers and touching anthers are depicted in figure 4. The most frequent visitors were queen *Bombus opifex* bumblebees, both in 2017 and 2018. Time separation between main pollinator guilds was remarkable (Fig. 5). Hummingbirds visited the flowers exclusively in the morning (8:00 to 10:00 h) and in the evening (19:00 to 21:00 h). By contrast, insects predominated in the afternoon, attaining a peak in visitation between 17:00 and 18:00 h.

We did not find significant differences between years (likelihood ratio test,  $F_{1,323} = 2.264$ ,  $P = 0.133$ ) nor between pollinator guilds (likelihood ratio test,  $F_{1,322} = 0.256$ ,  $P = 0.873$ ) in the number of consecutive flower visits within a plant. The median number of visited flowers was 6 for bees ( $n = 309$  individuals), while it was 5 for hummingbirds ( $n = 53$ ). However, when considering the four main flower visitors we found significant differences (likelihood ratio test,  $F_{3,320} = 10.339$ ,  $P < 0.0001$ ), while we did not detect a significant effect of year (likelihood ratio test,  $F_{1,323} = 2.687$ ,  $P = 0.102$ ). Median number of flowers visited within a plant were 5 for *Anthophora paranaensis* ( $n = 132$ ) and *Sappho sparganura* ( $n = 53$ ), 6 for *Bombus opifex* workers ( $n = 29$ ) and 9 for *B. opifex* queens ( $n = 111$ ).

#### DISCUSSION

*Salvia stachydifolia* displayed a wide range of reproductive strategies, from asexual reproduction (apomixis) to cross-pollination. Sexual phases and breeding system in *S. stachydifolia* suggest a dependence on pollinator activity. Nectar concentration in the studied population was typical of bumblebee-pollination (30-55 Brix %, Willmer 2011; Wester and Claßen-Bockhoff 2011),

but we detected two peaks of nectar concentration in the first day of flower life. Nectar volume did not differ from previous records for bee-pollinated *Salvia* species (Benitez-Vieyra *et al.* 2014). Bees were the most frequent visitors in the studied population, but hummingbirds dominated visits early in the morning and in the evening. Although hummingbirds are less frequent visitors, we did not test whether they were as efficient as bees in pollen delivery and deposition. Future works about the relative efficiency of different pollinators guilds can help to confirm if it was the case, using direct measures of pollinator efficiency, such as pollen deposition on stigmas, in order to identify the most effective pollinator in *S. stachydifolia*.

As other *Salvia* species (Cuevas García *et al.* 2013, Rosas Guerrero *et al.* 2017, Cairampoma *et al.* 2020), *S. stachydifolia* was partially protandrous, as pollen was exposed to pollinators before the beginning of stigma receptivity. Male phase was short and between 9:00 and 12:00 h, first-day flowers turned to the hermaphroditic phase, with more than 50% of the flowers displaying full receptivity. *Salvia stachydifolia* had an unusually short male phase, followed by a very long hermaphroditic phase in comparison with previous records in *S. elegans* (Rosas- Guerrero *et al.* 2017), but a short male phase duration was also recorded in the mixed-pollinated *S. rhombifolia* (Cairampoma *et al.* 2020). We did not find complete dichogamy, as expected in a bee-pollinated, self-compatible species. However, as all previous records of sexual phases in American *Salvia* came from hummingbird-pollinated (Cuevas García *et al.* 2013; Rosas Guerrero *et al.* 2017) or mixed-pollinated species (Cairampoma *et al.* 2020), we cannot test whether incomplete dichogamy in *S. stachydifolia* was the result of relaxed selection imposed by mixed pollination or a conserved character in the genus.

*Salvia stachydifolia* was self-compatible, like previous records in other *Salvia* species (Haque and Goshal 1981; Cuevas *et al.* 2018; Cuevas-García *et al.* 2013; Rosas Guerrero *et al.* 2017), but open pollination resulted in a three-fold increase in reproductive success in comparison with autonomous self-pollination, indicating that most of the seeds naturally produced in the population arise from pollinator activity. As main visitors (both bees and hummingbirds) visited five to six consecutive flowers per plant, this increase in reproductive success may be mainly the result of

geitonogamous pollination. Shifts to hummingbird pollination from bee-pollinated ancestors are more frequent in self-compatible lineages and that transition to a more efficient but less abundant pollinator is favored under a broadened set of ecological conditions if plants are capable of delayed selfing rather than obligate outcrossing (Wessinger and Kelly, 2018). This suggests that self-compatibility can be a preadaptation for certain types of pollinator transition or the evolution of pollinator specialization (Wessinger and Kelly 2018).

Nectar slowly accumulated along the first day of flower life, with two peaks of sugar concentration, early in the morning and in the afternoon, almost at the first peak of hummingbird activity and at the peak of bee activity, respectively (Fig. 2, 5). However, this bimodal pattern has to be taken with caution, as we only measured nectar dynamics in first-day flowers and under controlled environmental conditions. The presence of two concentration peaks even though volume increased in the same period indicates that two pulses of active sugar secretion happened during the first day of flower life (Galetto and Bernardello 2005). High nectar concentration and low volume are typical of bee-pollinated plants (Willmer, 2011; Wester and Claßen-Bockhoff 2011), but these features do not exclude hummingbirds, which often behave opportunistically (Espino-Espino *et al.* 2014). Inspections of the surrounding area indicated the absence of ornithophilous flowers, thus hummingbirds mostly depend on alternative local floral resources, like *S. stachydifolia*. In summary, most nectar traits fit the characteristics of a bee-pollinated species, opportunistically visited by hummingbirds.

Spatial and temporal variation in pollinator assemblages mean that the service provided by different pollinator guilds tends to vary from place to place and from time to time (Robertson *et al.* 2005). For instance, Castellanos *et al.* (2003) found that hummingbirds increase their visits to bee-syndrome flowers when they are at high densities or when there are low densities of alternative nectar sources, and can pollinate bee-syndrome flowers almost as well as bees do. Similarly, Cairampoma *et al.* (2020) found daily differences in pollinators activities in *Salvia rhombifolia* and showed that, despite traits associated with bee-pollination, *S. rhombifolia* has an intermediate pollination strategy in response to environmental conditions in which bees are disfavored. Scenarios that favor an increase in hummingbird visits may explain the numerous shifts in pollination syndrome in *Salvia* subgen.

*Calosphace* (Fragoso-Martínez *et al.* 2018, Sazatornil 2018) and in other Andean plant clades (e.g. Smith *et al.* 2008). Understanding if this increase is related to hummingbird habitat preferences would be a great step towards determining in which conditions one pollinator guilds become more efficient in pollinating *S. stachydifolia* and, in general, to understand the ecological circumstances in which pollination shifts occur. When circumstances encourage higher hummingbird visitation rates over a number of generations, adaptations to hummingbird pollination would be expected. After hummingbirds were acquired as reliable pollinators, selection may favor other traits that make the flowers more specialized for hummingbird pollination and discourage bee visits (i.e. reduced landing platforms, small flower entrance, long floral tubes, high nectar volume, low sugar concentration and absence of nectar guides), a pattern that has been recently reported in other *Salvia* species (Wester *et al.* 2020).

A recent review of the syndrome concept (Dellinger 2020) remarks that, despite of its enormous utility at macroevolutionary scales (with which we agree), we must be aware of the fact that any typological categorisation, such as functional groups and syndromes, leaves us at the boundaries of subjective interpretation of continuous, dynamic natural systems. Among the risks of this discretisation, it does not acknowledge populations' distinctive features, which are essential to understand the adaptive potential of populations or the direction/strength of selection (Dellinger 2020). *S. stachydifolia* is a highly variable species, particularly in flower shape and size (Wood 2007; O'Leary and Moroni 2016), thus it constitutes an ideal study system to test whether population differences in pollinator assemblages have generated divergent selective pressures which promoted these phenotypic differences and to understand the ecological process behind pollinator-mediated diversification in plants.

## FUNDING

This study was supported by FONCyT grant PICT 2017-2196 to SBV and by FONCyT grant PICT-2018-03192 to FS.

## ACKNOWLEDGEMENTS

We thank to Andrea Cocucci, Alicia Sérsic, Constanza Maubecin, Ana Clara Ibañez and Mauricio Cisternas for assistance during field work, to Juliana Izquierdo for valuable information and discussions about geographical variation in *S. stachydifolia* and to Agnes Dellinger for insightful discussions about syndrome concept in the final Manuscript.

Accepted Manuscript



## REFERENCES

- Abrahamczyk S & Renner SS (2015) The temporal build-up of hummingbird/plant mutualisms in North America and temperate South America. *BMC evolutionary biology* **15**: 104.
- Aigner PA (2001). Optimality modelling and fitness trade-offs: when should plants become pollinator specialists? *Oikos* **95**:177–184
- Aigner PA (2006). The evolution of specialized floral phenotypes in a fine-grained environment. Pages 23–46 in N. M. Waser and J. Ollerton, eds. Plant-pollinator interactions: from specialization to generalization. *University of Chicago Press, Chicago*.
- Cairampoma L, Tello JA, and Claßen-Bockhoff R (2020). Pollination in the desert—adaptation to bees and birds in *Salvia rhombifolia*. DOI: <https://doi.org/10.1086/710219>
- Castellanos MC, Wilson P, Thomson JD (2004) “Anti-bee” and “pro-bird” changes during the evolution of hummingbird pollination in *Penstemon* flowers. *Journal of Evolutionary Biology* **17**: 876–885.
- Castellanos MC, Wilson P, Thomson JD (2003) Pollen transfer by hummingbirds and bumblebees, and the divergence of pollination modes in *Penstemon*. *Evolution* **57**: 2742–2752.
- Cruden RW (1972) Pollinators in high-elevation ecosystems: relative effectiveness of bees and birds. *Science* **176**:1439–1440.
- Cuevas E, Espino J, Marques I (2018) Reproductive isolation between *Salvia elegans* and *S. fulgens*, two hummingbird-pollinated sympatric sages. *Plant Biology* **20**: 1075–1082.
- Cuevas-García E, Alcalá-Guerra A, Baños-Bravo Y E, Flores-Palacios A (2013) Biología reproductiva y robo de néctar en *Salvia gesneriflora* (Lamiaceae) y sus consecuencias en el éxito reproductivo. *Botanical Sciences* **91**: 357–362.
- Dafni A, Kevan PG, Husband BC (2005) Practical pollination biology. *Enviroquest Ltd. Cambridge, Canada*.

- Dellinger AS, Scheer LM, Artuso S, *et al.* (2019). Bimodal pollination systems in Andean Melastomataceae involving birds, bats, and rodents. *The American Naturalist* **194**, 104-116.
- Espino-Espino J, Rosas F, Cuevas-García E (2014) Variación temporal de visitantes florales en dos especies simpátricas de *Salvia* con floración simultánea y síndrome de polinización contrastante. *Revista mexicana de biodiversidad* **85**: 161-166.
- Fenster CB, Armbruster WS, Wilson P, *et al.* (2004) Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution and Systematics* **35**:375-403.
- Fragoso-Martínez I, Martínez-Gordillo M, Salazar GA, *et al.* (2018) Phylogeny of the Neotropical sages (*Salvia* subg. *Calosphace*; Lamiaceae) and insights into pollinator and area shifts. *Plant Systematics and Evolution*, **304**: 43–55.
- Galetto L, Bernardello G (2005) Nectar. In A. Dafni, P. G. Kevan, & B. C. Husband (Eds.), *Pollination ecology: a practical approach*. (pp. 156–212). Ontario, Canada: Enviroquest Ltd.
- Grant KA & Grant V (1968) *Hummingbirds and their flowers*. *Columbia University Press*.
- Haque M S, Ghoshal KK (1981). Floral biology and breeding system in the genus *Salvia* L. *Proceedings of the Indian Natural Sciences Academy* **47**:716-724.
- Harder LD, Jordan CY, Gross WE, Routley MB. (2004). Beyond floricism: the pollination function of inflorescences. *Plant Species Biology*, **19(3)**: 137-148.
- Knox RB, Williams EG, Dumas C (1986) Pollen, pistil and reproductive function in crop plants. *Plant Breed Rev* **4**:9–79
- Kriebel R, Drew BT, Drummond, C P, *et al.* (2019). Tracking temporal shifts in area, biomes, and pollinators in the radiation of *Salvia* (sages) across continents: leveraging anchored hybrid enrichment and targeted sequence data. *American Journal of Botany*, **106(4)**, 573-597.
- Lenth R (2018). *emmeans*: Estimated Marginal Means, aka Least Squares Means. *R package*. See <https://CRAN.R-project.org/package=emmeans>.

- Manning, JC & Goldblatt P (2005). Radiation of pollination systems in the Cape genus *Tritoniopsis* (Iridaceae: Crocoideae) and the development of bimodal pollination strategies. *International Journal of Plant Sciences* **166**:459–474.
- Muchhala N (2007) Adaptive trade-off in flower morphology mediates specialization for flowers pollinated by bats and hummingbirds. *American Naturalists*. **169**: 494–504.
- Pérez F, Arroyo MTK, Medel R, MA Hershkovitz MA (2006). Ancestral reconstruction of flower morphology and pollination systems in *Schizanthus* (Solanaceae). *American Journal of Botany* **93**:1029-1038.
- O’Leary N, Moroni P (2016). Las especies de *Salvia* (Lamiaceae) para Argentina. *Darwiniana* **4**: 91-131.
- R Core Team (2019) R: A language and environment for statistical computing. *R Foundation for Statistical Computing*, Vienna, Austria. URL <https://www.R-project.org/>.
- Robertson AW, Ladley JJ, Kelly D (2005). Effectiveness of short-tongued bees as pollinators of apparently ornithophilous New Zealand mistletoes. *Austral Ecology*, **30(3)**: 298-309.
- Rosas-Guerrero V, Aguilar R, Martín-Rodríguez S, *et al.* (2014) A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecology Letters* **17**: 388–400.
- Rosas-Guerrero V, Hernández D, Cuevas E (2017). Influence of pollen limitation and inbreeding depression in the maintenance of incomplete dichogamy in *Salvia elegans*. *Ecology and Evolution* **7**: 4129–4134.
- Ruiz Zapata T, Arroyo MT (1978) Plant reproductive ecology of a secondary deciduous tropical forest in Venezuela. *Biotropica* **10**:221-230.

- Salas-Arcos L, Lara C, Castillo-Guevara C, *et al.* (2019) “Pro-bird” floral traits discourage bumblebee visits to *Penstemon gentianoides* (Plantaginaceae), a mixed-pollinated herb. *The Science of Nature*, **106**: 1.
- Sargent RD, Otto SP. (2004). A phylogenetic analysis of pollination mode and the evolution of dichogamy in angiosperms. *Evolutionary Ecology Research*, **6(8)**:1183-1199.
- Sazatornil FD (2018). Cambios en el fenotipo floral de especies neotropicales de *Salvia* asociados a transiciones en el síndrome de polinización: Integración de patrones filogenéticos, microevolutivos y herramientas de morfometría geométrica. PhD thesis. *Facultad de Ciencias Exactas Físicas y Naturales, Universidad Nacional de Córdoba*.
- Smith SD, Ané C & Baum DA. (2008). The role of pollinator shifts in the floral diversification of *Ioichroma* (Solanaceae). *Evolution: International Journal of Organic Evolution*, **62(4)**:793-806.
- Strelin MM, Sazatornil F, Benitez-Vieyra S, Ordano M. (2017) Bee, hummingbird or mixed pollinated *Salvia* species mirror pathways to pollination optimization: a morphometric analysis based on the Pareto front concept. *Botany* **95**:139-146.
- Thomson JD (2003). When is it mutualism? *American Naturalist* **162**(suppl.):S1–S9.
- Thomson JD & Wilson P (2008) Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence, and directionality. *International Journal of Plant Sciences*, **169**: 23-38.
- Van der Pijl L (1961) Ecological aspects of flower evolution. II. Zoophilous flower classes. *Evolution*, **15**: 44-59.
- Wessinger CA, Kelly JK (2018). Selfing can facilitate transitions between pollination syndromes. *The American Naturalist*, **191(5)**: 582-594.

- Wester P, Claßen-Bockhoff R (2011) Pollination syndromes of New World *Salvia* species with special reference to bird pollination. *Annals of the Missouri Botanical Garden* **98**:101-155.
- Wester P, Cairampoma L, Haag S, Schramme J, Neumeyer C, & Claßen-Bockhoff R (2020). Bee exclusion in bird-pollinated *Salvia* flowers – the role of flower colour versus flower construction. *International Journal of Plant Sciences*. DOI: <https://doi.org/10.1086/709132>
- Willmer P (2011) Pollination and floral ecology. *Princeton University Press*.
- Wood JRI (2007) The *Salvias* (Lamiaceae) of Bolivia. *Kew Bulletin* **62**, 177-221.
- Wood SN (2006) Generalized Additive Models: An Introduction with R. *Chapman and Hall/CRC Boca Raton*.
- Wood SN (2008). Fast stable direct fitting and smoothness selection for generalized additive models. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)* **70**: 495-518.
- Wood S, Scheipl F (2017). Package 'gamm4': Generalized Additive Mixed Models using 'mgcv' and 'lme4'. Version 0.2-5. *Published*, 25-07-2017.

Accepted Manuscript

TABLES

**Table 1.** Flower visitors and visitation rates to *Salvia stachydifolia*.

Pollinators	visits·flower <sup>-1</sup> ·h <sup>-1</sup>	Visits %
Hymenoptera:Apidae <i>Bombus opifex</i> (queens)	0.294	46%
Hymenoptera:Apidae <i>Anthophora paranaensis</i>	0.132	21%
Hymenoptera:Apidae <i>Bombus opifex</i> (workers)	0.078	12%
Other Hymenoptera	0.040	6%
Trochiliformes:Trochilidae <i>Sappho sparganura</i>	0.074	12%
Diptera:Bombyliidae unknown sp.	0.019	3%

## FIGURE CAPTIONS

**Figure 1.** Pattern of anthesis and sexual phases in *Salvia stachydifolia*. a) Number of open flowers and flowers with dehiscent anthers along seven days in a cohort of 70 flowers. Different anther phases (closed and dehiscent) are indicated by continuum and dotted lines, respectively. b) Proportion of receptive flower stigmas during three different days, at different hours. Each column summarises results from five to eight flowers.

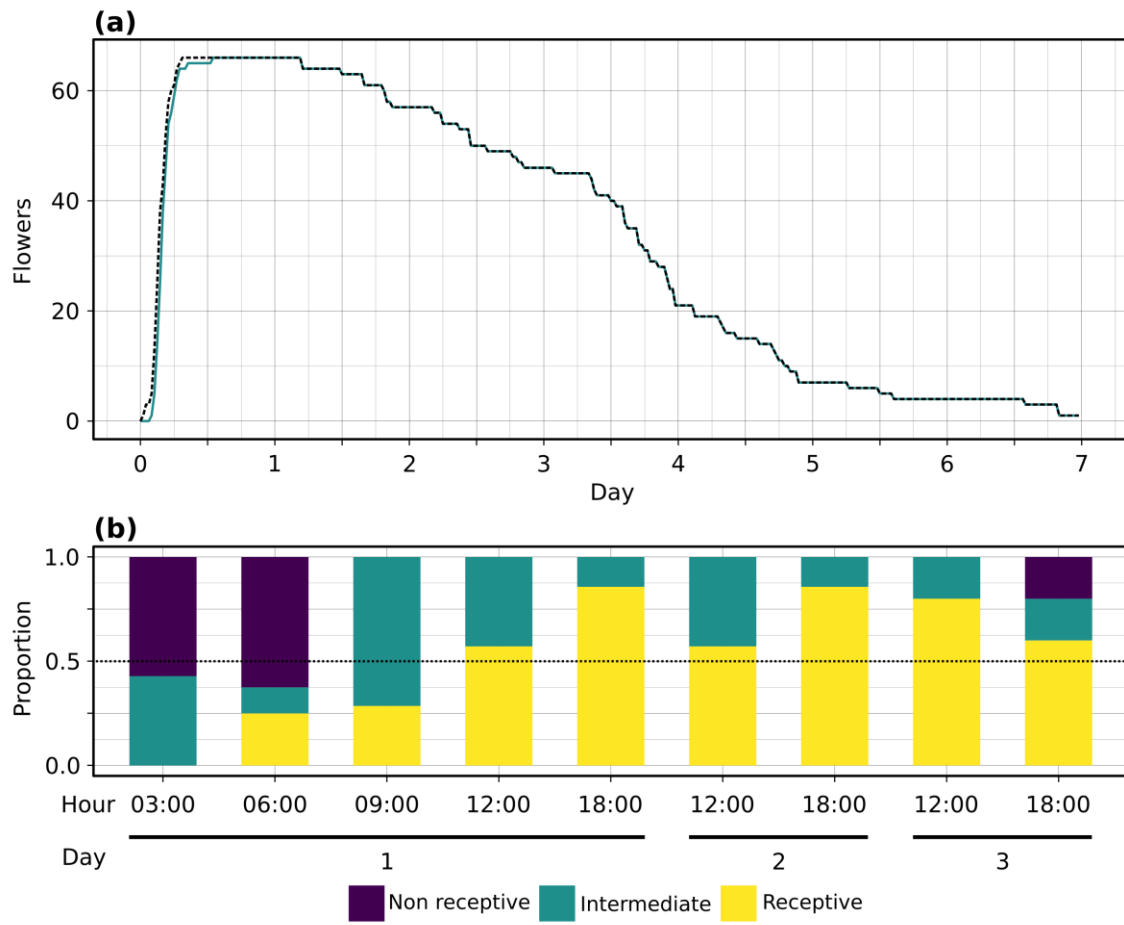
**Figure 2.** Nectar dynamics in *Salvia stachydifolia*. Generalized additive mixed models were performed to show the dynamics through time of a) concentration and b) volume. Dotted lines indicate one Bayesian standard error.

**Figure 3.** Reproductive success under five pollination treatments in *Salvia stachydifolia*. a) Fruit set (proportion of flowers that set fruits) and b) seed set per flower (average number of seeds per flower). Five pollination treatments were abbreviated as follows: **ap** (apomixis, n= 31), **as** (autonomous self-pollination, n= 35), **hs** (hand self-pollination, n= 32), **hc** (hand cross-pollination, n= 36) and **op** (open pollination, n= 30). Mean values sharing the same letter were not significantly different according to a Tukey *post hoc* test. Standard errors are indicated.

**Figure 4.** Main visitors in *Salvia stachydifolia*, visiting and touching reproductive organs of the flowers a) *Sappho sparganura*, b) *Bombus opifex* queen.

**Figure 5.** Visitation rates through time in *Salvia stachydifolia*. Colours indicate hummingbird visitation rate (dark grey bars) and bees and flies visitation rates (light grey bars). Vertical lines indicate one standard error.

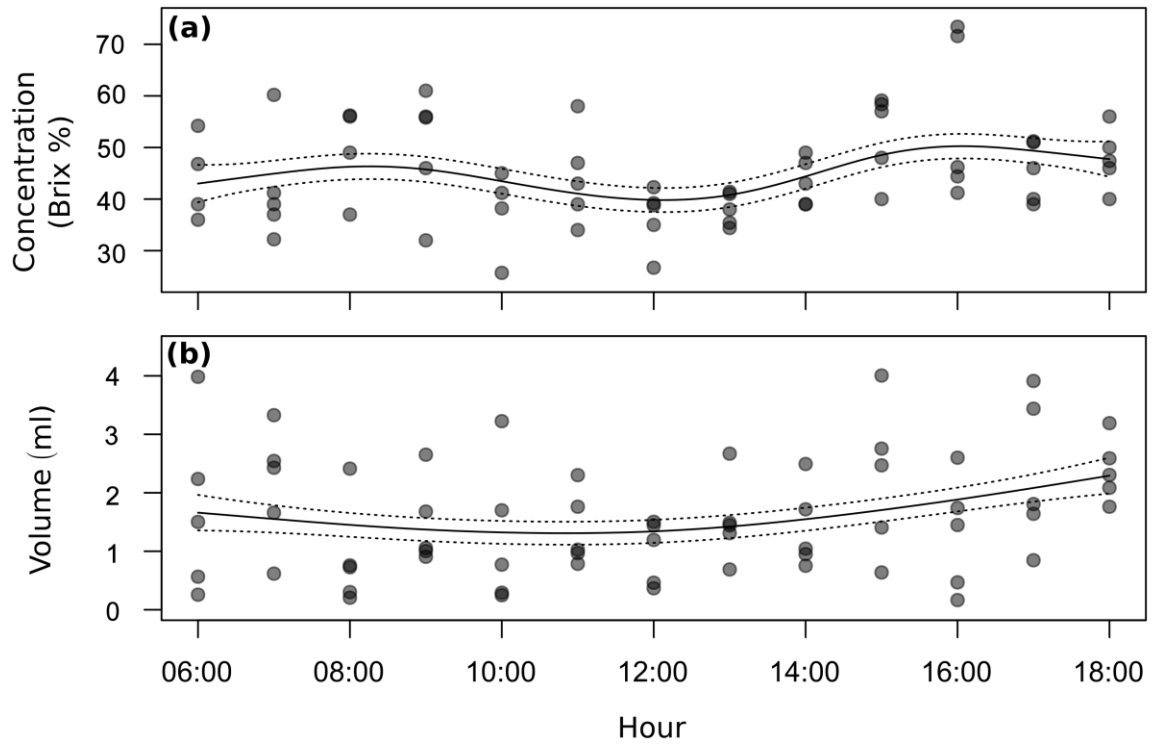
Figure 1



Accepted

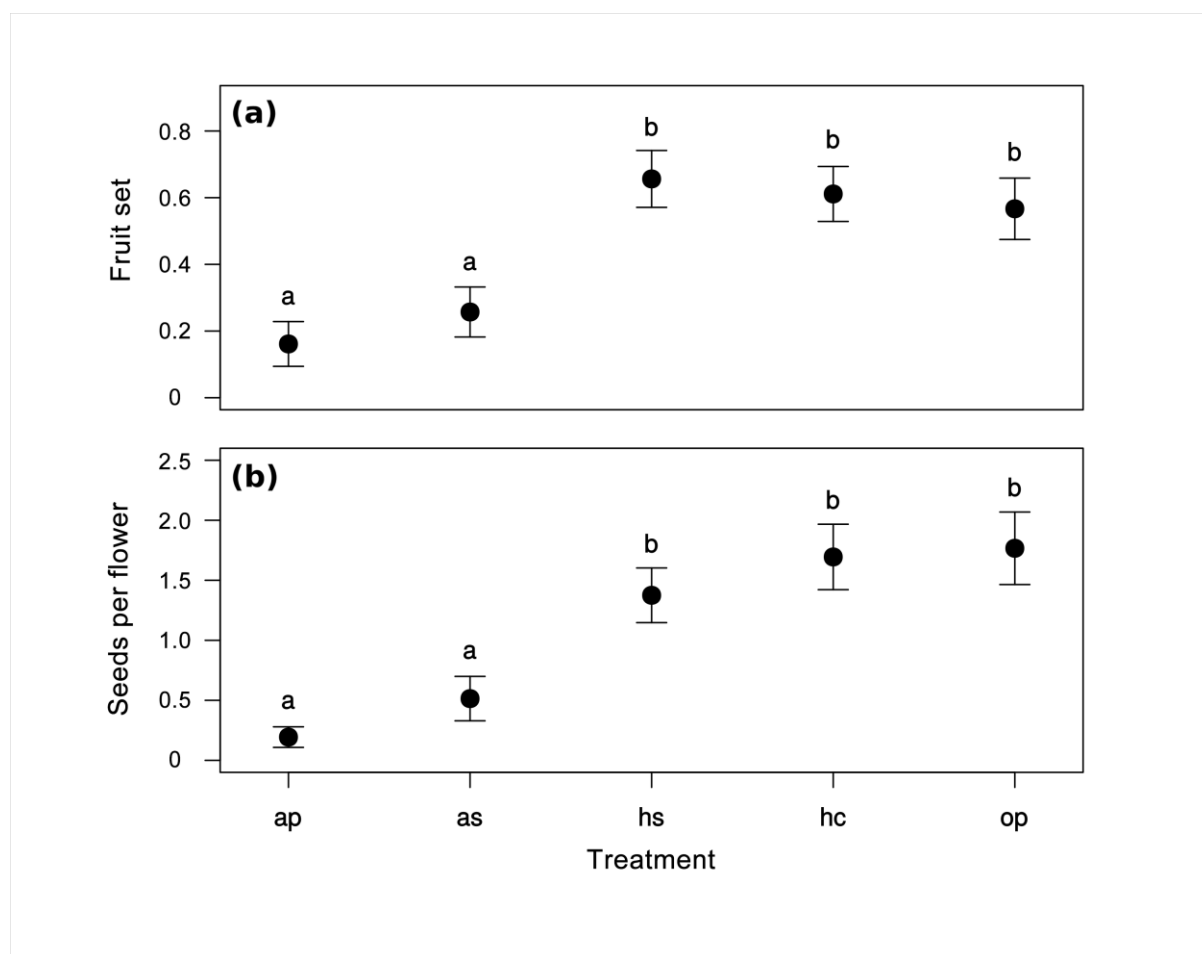


Figure 2



Accepted Manuscript

Figure 3



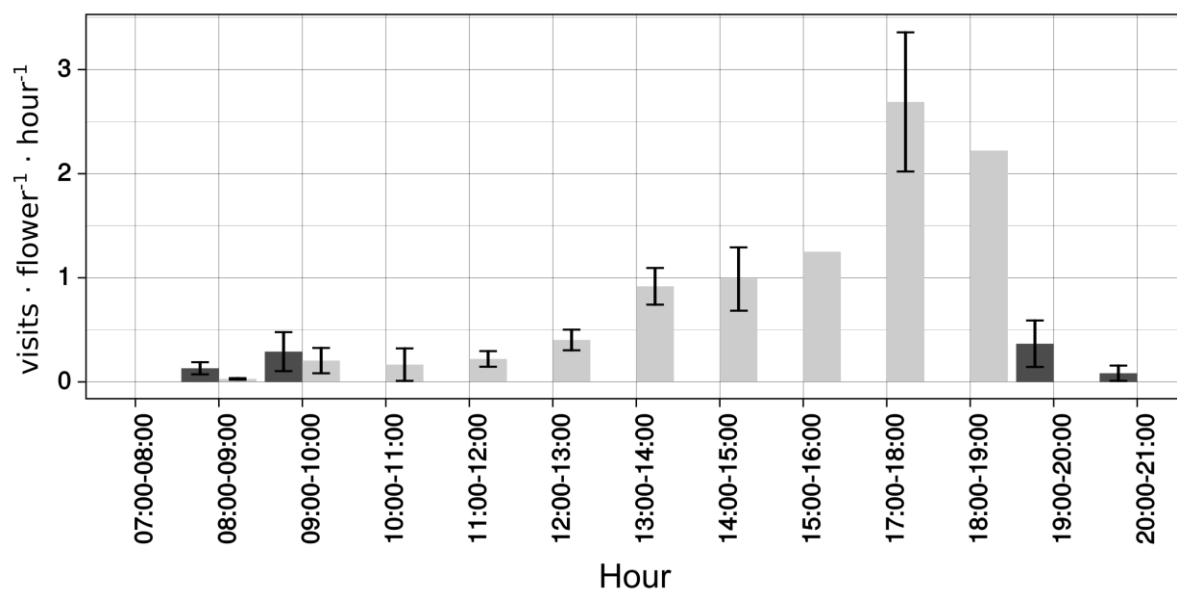
Accepted

Figure 4



Accepted Manuscript

Figure 5



Accepted Manuscript