

Stigma shape shifting in sages (*Salvia*: Lamiaceae): hummingbirds guided the evolution of New World floral features

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A fundamental question in evolutionary biology is how clades of organisms exert influence on one another. The evolution of the flower and subsequent plant/pollinator coevolution are major innovations that have operated in flowering plants to promote species radiations at a variety of taxonomic levels in the Neotropics. Here we test the hypothesis that pollination by Neotropical endemic hummingbirds drove the evolution of two unique stigma traits in correlation with other floral traits in New World *Salvia* (Lamiaceae). We examined morphometric shapes of stigma lobing across 400 *Salvia* spp., scored presence and absence of a stigma brush across *Salvia*, and used a suite of phylogenetic comparative methods to detect shape regime shifts, correlation of trait shifts with BayesTraits and phylogenetic generalized least square regressions, and the influence of scored pollinators on trait evolution using OUwie. We found that a major Neotropical clade of *Salvia* evolved a correlated set of stigma features, with a longer upper stigma lobe and stigmatic brush, following an early shift to hummingbird pollination. Evolutionary constraint is evident as subsequent shifts to bee pollination largely retained these two features. Our results support the hypothesis that hummingbirds guided the correlative shifts in corolla, anther connective, style and stigma shape in Neotropical *Salvia*, despite repeated shifts back to bee pollination.

ADDITIONAL KEYWORDS: evolutionary constraint – evolutionary shifts – morphometrics – pollination – secondary pollen presentation – stigma morphology.

INTRODUCTION

The extraordinary adaptive radiation of angiosperms beginning in the mid-Mesozoic and continuing throughout the Cenozoic is inherently connected to the evolution of the flower. The evolution of the flower and subsequent plant/pollinator coevolution are major

innovations that have operated in flowering plants to promote species radiations at a variety of taxonomic levels (Darwin, 1862; Grant, 1949; Stebbins, 1970; Claßen-Bockhoff *et al.*, 2004; van der Niet, Peakall & Johnson, 2014; Lagomarsino *et al.*, 2017; Smith & Kriebel, 2018; Kriebel *et al.*, 2020). The importance of pollinator-mediated selection on floral traits has been demonstrated through field (e.g. Whittall & Hodges, 2007), genetic (e.g. Opedal, 2019) and comparative

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phylogenetic studies (e.g. Smith, 2010; Smith & Kriebel, 2018; Kriebel *et al.*, 2020).

Flowering plants have evolved remarkable variation in floral traits and breeding systems as they have adapted to the changing landscapes of new areas, biomes and their attendant animal pollinators (Sprengel, 1793; Lloyd & Barrett, 1996; Harder & Johnson, 2009; Barrett & Harder, 2017). This diversification is especially notable in the Neotropics where radiations in numerous plant clades are intimately associated with endemic hummingbird or bumble bee pollinators (e.g. Berry *et al.*, 2004; Kay *et al.*, 2005; Tripp & McDade, 2013; Abrahamczyk & Renner, 2015; Iles *et al.*, 2017; Serrano-Serrano *et al.*, 2017; Kessler, Abrahamczyk & Krömer, 2020). The study of how pollinators influence floral evolution has typically relied on relatively conspicuous discrete components of plant morphology such as inflorescence arrangement, corolla colour, general floral shape, and size and relative positions of the anther and stigma (Dellinger, 2020). However, phylogenetic comparative methods are now using continuous morphometric data to study the interactions between flowers and pollinators. Most of these studies have focused on the corolla (e.g. Smith & Kriebel, 2018), although recently the anther and style have been examined (Vasconcelos *et al.*, 2019; Kriebel *et al.*, 2020). However, phylogenetic comparative studies focusing on the evolution of the stigma or style lobes with morphometric data have been strikingly absent.

The style and stigma function primarily in their roles of pollen reception, recognition, rehydration and mediating pollen tube migration into the ovary (Edlund, Swanson & Preuss, 2004; Basso-Alves, Agostini & de Pádua Teixeira S, 2010), but they also can serve additional roles in pollination biology that can influence plant diversification. First, the length, position and timing of maturation of the style through dichogamy, herkogamy, heterostyly and enantiostyly play important roles in outcrossing (Webb & Lloyd, 1986; Bertin & Newman, 1993; Barrett, Jesson & Baker, 2000a; Barrett, Wilken & Cole, 2000b; Barrett, 2002a; Jesson & Barrett, 2002). Flexible styles can change positions (flexistily), improving the efficiency of protandry, increasing outcrossing or enabling delayed selfing (Li *et al.*, 2001; Ruan *et al.*, 2010). In addition, secondary pollen presentation (SPP), in which the pollen within a flower is presented by a structure in addition to the anther, is often carried out by the style or stigmatic lobes (Howell, Slater & Know, 1993; Yeo, 1993; Ladd, 1994). The success of several large groups (e.g. Asteraceae and Proteaceae) has been linked to SPP and the use of stigmatic lobes (Leins & Erbar, 2006). Similarly, a stylar brush acting as an SPP, with or without a pumping mechanism, has

evolved repeatedly in angiosperms (e.g. Nyman, 1993; Kang & Zhang, 2009; Erbar & Leins, 2015). Finally, the evolution to bilobed style/stigmas has facilitated changes involved in pollination (Milet-Pinheiro *et al.*, 2009; Wright, Welsh & Costea, 2011) and may have acted as a selective agent favouring shifts to a wider range of pollinators (Katinas *et al.*, 2016).

Salvia L. (sages, Lamiaceae), a genus with c. 1000 species, offers a unique opportunity to examine the evolution of the stigma as it exhibits considerable shape and size diversity in stigma lobing and shows variation in the presence or absence of a stylar brush (Fig. 1). *Salvia* is distributed across the Old World (OW) and New World (NW), occurs in several different biome types, is pollinated by bees and birds (Wester & Claßen-Bockhoff, 2006b, 2007, 2011; Celep *et al.*, 2020; Barrionuevo, Benitez-Vieyra & Sazatornil, 2021), displays striking variation in other floral traits and has undergone four significant shifts in species diversification (Kriebel *et al.*, 2019, 2020). Importantly, a well-sampled phylogenetic framework based on sequences from both anchored hybrid enrichment and traditional targeted sequencing is available for *Salvia* (Drew *et al.*, 2017; Kriebel *et al.*, 2019; Rose *et al.*, 2021). Previous studies have provided a wealth of data on floral morphology in the context of pollination biology in *Salvia*. These data permit the formulation of specific hypotheses we test here. Wester & Claßen-Bockhoff (2006a) demonstrated that the styles in some hummingbird-pollinated species in *Salvia* subgenus *Calosphace* (Benth.) Epling were straighter than those in bee-pollinated species from both the OW and NW. Recent morphometric analyses of the shape of anther connectives and styles (minus stigmatic lobes) indicate that the evolution from curved to straight anthers and styles occurred early in the NW subgenus *Calosphace* and near the edge with the main shift from bee to hummingbird pollination c. 20 Mya (Kriebel *et al.*, 2020), a timing consistent with the radiation of hummingbirds (McGuire *et al.*, 2014). Subsequent reversals back to bee pollination in subgenus *Calosphace* were not linked to changes in anther or style shape and suggested a strong evolutionary constraint imposed on anther connective and style shape by the original shift in floral form in response to hummingbird pollination (Kriebel *et al.*, 2020).

Finally, El-Gazzar *et al.* (1968) showed with limited sampling that species in this mostly straight-styled clade of subgenus *Calosphace* possessed asymmetric stigmatic lobes with the upper longer than the lower lobe (see figs 1B–D and 4 of El-Gazzar *et al.* 1968) and hypothesized that this morphology was derived and diagnostic of Mexican and Central/South American sages. The preliminary study of El-Gazzar and colleagues demonstrated that the 35 species examined

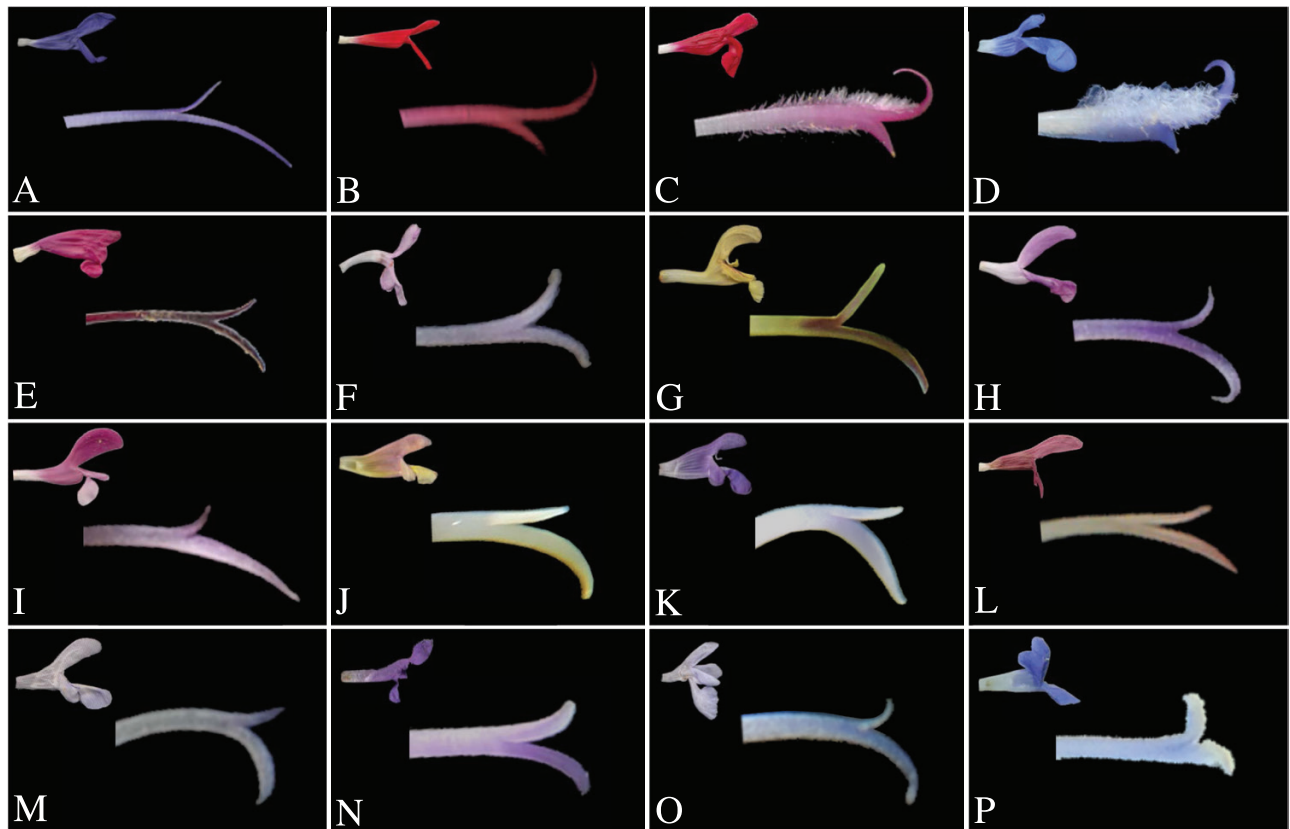


Figure 1. Examples of stigmas in different subgenera of *Salvia* (classification follows Drew *et al.*, 2017, Kriebel *et al.*, 2019) with geographical distribution and pollinator of each species indicated in parentheses (NW, New World; OW, Old World). A corolla of each species is placed on the upper left-hand corner for reference. Subgenus *Calosphace*: A, *S. guaranitica* (NW bird); B, *S. regla* (NW bird); C, *S. greggii* (NW bird); D, *S. prunelloides* (NW bee). Subgenus *Audibertia*: E, *S. spathacea* (NW bird); F, *S. leucophylla* (NW bee). Subgenus *Glutinaria*: G, *S. omeiana* (OW bee). Subgenus *Sclarea*: H, *S. canariensis* (OW bee); I, *S. hierosolymitana* (OW bee). Subgenus *Salvia*: J, *S. adenophylla* (OW bee); K, *S. officinalis* (OW bee). Subgenus '*Heterosphace*': L, *S. lanceolata* (OW bird); M, *S. somalensis* (OW bee); N, *S. verticillata* (OW bee). Subgenus *Rosmarinus*: O, *S. rosmarinus* (OW bee). Subgenus *Perovskia*: P, *S. yangii* (OW bee). Corollas and stigmas not to scale.

from subgenus *Calosphace* differed from the other 65 sampled in having the upper stigmatic lobe longer than the lower one (vs. shorter or equal). They also documented and illustrated (El-Gazzar *et al.*, 1968; El-Gazzar & Watson, 1970) the presence of a stigmatic brush (i.e. brush-like trichomes on the style just below the stigma) in 28 of these 35 species with a longer upper stigmatic lobe (e.g. Fig. 1C) and the absence of the stigmatic brush in a species of OW subgenus *Salvia* with a longer lower style lobe (e.g. Fig. 1H). The functions of these two unique features of stigmatic lobing and brush have, however, not been clarified, and these stigma traits have not been examined subsequently in any study of *Salvia*.

We implement one of the first extensive evaluations of stigma shape evolution in flowering plants to specifically test hypotheses regarding the evolution of these characters in the context of two different types of Neotropical pollinators (hummingbirds and

bees). We couple morphometric data of stigmas of *Salvia* quantified using elliptic Fourier analysis with pollinator data for nearly 400 species. Phylogenetic comparative analyses of these data are implemented with Ornstein–Uhlenbeck (OU) process models using the *l1ou* and *OUwie* packages, phylogenetic generalized least square (PGLS) regressions and BayesTraits across a phylogenetic tree including over half of the species in *Salvia*. Hypotheses of correlated evolution and specific contingent evolution are tested using three traits: pollinator, stigmatic lobing and stigmatic brush. We test the following hypotheses: (1) stigmas with the upper stigmatic lobe longer than the lower one and possessing a stigmatic brush are confined to the NW and largely Neotropical subgenus *Calosphace* (El-Gazzar *et al.*, 1968); (2) these two stigmatic traits are contingent on the origin of hummingbird pollination in subgenus *Calosphace*; (3) these two stigmatic traits display evolutionary constraint and are

maintained despite reversals back to bee pollination as demonstrated with other floral features (Kriebel *et al.*, 2020); and (4) these two stigmatic traits evolved at a similar time and in correlated manner with a suite of floral traits (corolla, anther connective and style shape) resulting from hummingbird pollination that pre-dated a major shift in species diversification in *Salvia* (Kriebel *et al.*, 2020).

MATERIAL AND METHODS

MORPHOMETRIC ANALYSES OF STIGMA SHAPE IN *SALVIA*

A Canon LiDE 220 (LED indirect exposure) flatbed scanner was used to scan entire flowers and their dissected structures (see Fig. 1 for examples). The scanned styles and stigmas were obtained from field collections and cultivated specimens from the University of California Berkeley, Denver and Rancho Santa Anna Botanical Gardens, and University of Wisconsin-Madison Botany Greenhouses. This dataset was supplemented by illustrations of *Salvia* stigmas from the literature. The 402 *Salvia* species sampled represented all major subgeneric clades (Drew *et al.*, 2017; Kriebel *et al.*, 2019). Floral scans were also obtained from two species of *Melissa* L. and six species of *Lepechinia* Willd. used as a monophyletic outgroup (Drew & Sytsma, 2012) for a total of 410 species. For further details of sampling and vouchers, see Kriebel *et al.* (2019).

Stigma shapes from *Salvia* and outgroups were then outlined, filled with black and placed on white backgrounds using GIMP 2.8 (Solomon, 2009; <http://www.gimp.org>). Elliptic Fourier analysis (eFa) was then performed with the package Momocs v.1.3.0 (Claude, 2008; Bonhomme *et al.*, 2014) in the R statistical environment (R Core Team, 2018). This approach was used recently to quantify the shape of corollas, anther connectives and styles in *Salvia* (Kriebel *et al.*, 2020), and further details are available there. Four landmarks were used to align the specimens: one on the base of stigma, one at the base of the stigma lobes where they diverge from each other and one at the apex of each stigmatic lobe. Examples of landmarked stigmas and stigmas landmarked and saved as an rds file and in jpg format are provided (Supporting Information, Fig. S1; Data Repository doi:10.5061/dryad.v15dv41v1). The landmarked outlines were aligned with a full generalized Procrustes alignment in Momocs before the eFa.

STIGMA VARIATION IN *SALVIA* RELATIVE TO BIOGEOGRAPHY AND POLLINATOR AND SUBCLADE

We first implemented a series of tests to examine whether stigma variation in morphospace is

significantly different when comparing species binned by biogeography, clade and pollinator. First, to compare stigma morphology relative to biogeographical area, all sampled *Salvia* spp. were assigned to the NW or OW based on Kriebel *et al.* (2019). Second, NW species were further divided into four clades: core-*Calosphace* [here used to include all species in the subgenus except the *Hastatae* clade + *S. axillaris* Moc. & Sessé ex Benth. and equal to clade E in Walker & Sytsma, 2007), the *Hastatae* clade of subgenus *Calosphace* (including sections *Blakea*, *Hastatae* and *Standleyana*, equal to clade F in Walker & Sytsma, 2007), subgenus *Audibertia* J.B.Walker, B.T.Drew & Sytsma (Walker, Drew & Sytsma, 2015), and the informal 'subgenus' *Heterosphace* (Kriebel *et al.*, 2020)]. Third, species were assigned to bee, bird or polymorphic bins based on Kriebel *et al.* (2020). Fourth, to examine possible evolutionary constraint in stigmas of bee-pollinated species derived from bird ancestry in the NW subgenus *Calosphace*, bee species were further separated into two bins, bee and bee derived from bird ancestry, following Kriebel *et al.* (2020). The area of morphospace occupied by each group and their overlap was quantified by calculating the convex hull around each set of points using the hull function in R, then converting each convex hull into a polygon, and finally using the *st_area* and *st_intersection* functions of the R package *sf* v.0.7-3 (Pebesma, 2018). The significance of mean differences between groups was evaluated with approximate randomization tests using the *independence_test* function of the R library *coin* v.1.3-0 (Hothorn *et al.*, 2008) resampling 10 000 times and the function *pairwisePermutationTest* in the R package *rcompanion* v.2.2.1 (Mangiafico, 2019).

We explored the evolution of stigmas in *Salvia* in an explicit phylogenetic context by using the maximum clade credibility (MCC) time-calibrated tree of 528 *Salvia* spp. from Kriebel *et al.* (2019). Species without stigma shape data were dropped from the MCC tree to create a reduced tree of 283 tips. To increase the exploration of tree space for examining stigma shape evolution and its correlates, we obtained 11 trees by summarizing results across the MCC tree and the median tree (also reduced to 283 species) from each of ten sets of grove trees obtained with the R package *treespace* (Kendall & Colijn, 2016) from the posterior probability distribution of the BEAST (Bouckaert *et al.*, 2014) analysis of Kriebel *et al.* (2019). The phylogenetic signal of the resulting principal components PC1 and PC2 of the stigma morphometric analysis was tested across this set of 11 trees using *phylosig* from the R library *phytools* v.0.7-18 (Revell, 2012) with both *lambda* (Pagel, 1999) and *K* (Blomberg, Garland & Ives, 2003) values.

Finally, we tested for differences in stigma morphology (PC1 and PC2) between species from

different geographical areas and with different pollinators by accounting for similarities in trait values due to common evolutionary history using PGLS regression (Grafen, 1989; Symonds & Blomberg, 2014) with the R library nlme v.3.1-139 (Pinheiro *et al.*, 2019). Models in which the correlation structure followed a Brownian motion (BM) (Felsenstein, 1985; Martins & Hansen, 1997), 'lambda' (Pagel, 1999) or OU (Hansen, 1997; Butler & King, 2004) process were first compared using likelihood ratio tests with the R package ape v.5.0 (Paradis & Schliep, 2018). The variance explained by each model was assessed using the coefficient of determination (R^2) calculated with the R library rr2 v.1.0.2 (Ives & Li, 2018; Ives, 2019). Phylogenetic sensitivity analyses were performed for PGLS models with the R package sensiPhy v.0.8.4 (Paterno, Penone & Werner, 2018). The function tree_clade_phylm was used to calculate the impact of influential clades on PGLS model estimates and incorporating phylogenetic uncertainty across the 11 trees.

SHIFTS IN STIGMA SHAPE EVOLUTION IN *SALVIA* MODELLED WITH THE OU PROCESS

The OU process was implemented to model the dynamics of stigma shape evolution and identify significant shape shifts during the diversification of *Salvia*. The R library l1ou v.1.42 was utilized to test for the best shift configuration in stigma shape with PC1, which explained the general shape of the stigma lobes across each tree. The phylogenetic Bayesian information criterion (pBIC) model was used as it is conservative when searching for regime shifts and avoids favouring overly complex models (Khabbazian *et al.*, 2016). A test for convergent evolution in stigma shape using l1ou was performed to identify convergent shape regimes across all trees.

To compare resulting regime changes in stigma shape with changes in pollination syndrome and switches in the presence or absence of the stigmatic brush, marginal ancestral states for both traits were estimated for every node on the set of reduced (283 species) trees using maximum likelihood (ML) with the R library corHMM v.1.22 (Beaulieu, Oliver & O'Meara, 2017). The reconstructions of each of these traits were done independently. The stigmatic brush of each *Salvia* sp. was categorized as either absent or present based on field observations, floral scans, photographs and the literature. The equal rates, symmetric, all rates different and two ordered models were tested using the fitMK function of the R library phytools (Revell, 2012) and compared with the Akaike information criterion (AIC). Estimated transition rates were used to set the weights at the root (Yang, 2006; Beaulieu, O'Meara

& Donoghue, 2013; Beaulieu *et al.*, 2017). Finally, a multivariate l1ou analysis was implemented by combining the stigma shape morphometric data with morphometric data previously obtained (Kriebel *et al.*, 2020) for corolla, anther connective and style shapes using PC1 on a reduced 172-species tree containing only species with all four data points present.

TESTS OF CORRELATIVE AND CONTINGENT EVOLUTION OF POLLINATOR, SUB-STIGMATIC BRUSH AND STIGMATIC LOBING TRAITS

We implemented a set of analyses using sub-stigmatic brush and stigma shape as discrete traits in addition to pollinator type across the suite of trees to examine the possible correlative or contingent evolution of pollinator, stigmatic brush and stigmatic lobing traits in *Salvia*. As many of the tests involved evolution occurring in *Salvia* subgenus *Calosphace*, we conducted an identical second set of analyses but on a reduced tree and dataset comprising only subgenus *Calosphace* and its sister clade subgenus *Audibertia*. BayesTraits 3.0.2 (Pagel & Meade, 2016) was used as implemented elsewhere for floral traits (e.g. Friedman & Barrett, 2008; Drew & Sytsma, 2013; Givnish *et al.*, 2014). Analyses utilized ML with scaletrees = 0.1 as recommended to avoid issues with rates approaching zero and maximum likelihood tries = 20 to ensure effective ML searching. Pollinator and brush were coded as binary (0, 1): pollinator (bee, bird), brush (absent, present). The few polymorphic pollinated species and polymorphic brush species in the NW were given a dash, which BayesTraits recognizes as either state. We utilized the results of both the PC stigma shape variation and the l1ou shift regimes in *Salvia* to discretize stigma shape that was captured in the morphometric analyses. PC1 describes stigma shape variation between upper lobe longer to lower lobe longer with a few species exhibiting equal lobing (Supporting Information, Fig. S1). The significant shift regime seen with l1ou also occurs at the transition to a longer upper stigmatic lobe from the background state of lower lobe longer or equal (Fig. 3). Thus, we were able to code stigmatic lobing across all 528 species in a binary fashion as either (0) upper lobe not longer (including equal lobed and longer lower lobed stigmas) or (1) upper lobe longer. Living floral material, floral scans, herbarium specimens and the literature were used to score this trait.

First, the function Multistate was used to test for significant differences in transition rates between the two states of each trait with the likelihood ratio test on the ML scores of the unconstrained and constrained ($q_{01} = q_{10}$) runs. Second, the function Discrete was implemented to test for correlative evolution of each

pair of characters (three tests) by using the likelihood ratio test on the ML scores of the independent model (four q transition rates) and the dependent model (eight q transition rates) with four degrees of freedom (4DF). The function Discrete provides the root state probabilities of paired correlated characters and the relative transition rates of each of eight possible transitions (e.g. q_{12} = transition rate from bee pollination and longer lower stigma lobe to bee pollination and longer upper stigma lobe; Fig. 5). Third, Discrete was used to examine specific tests of contingency (see Table 1 for list). For example, we predict that longer upper stigma lobes are correlated with the stigma brush; thus, can the null model 'transitions from the longer lower stigma lobes to longer upper stigma lobes occur at the same rate in clades with and without the stigma brush' be rejected? To implement this test, we restricted the two transitions as being equal ($q_{13} = q_{24}$; see Fig. 5C) and performed a likelihood ratio test on the ML scores of this contingent model (seven q transition rates) against the full dependent model (eight q transition rates) with one degree of freedom. If the test is significant, the null model is rejected in favour of contingent evolution favouring the evolution of longer upper stigma lobes in clades possessing stigma brushes (if $q_{24} > q_{13}$).

EVOLUTIONARY CONSTRAINT IN STIGMA TRAITS IN *SALVIA*

Finally, we tested whether evolutionary constraint was limiting changes in stigma shape in subgenus *Calosphace* after the early shift to hummingbird pollination (Kriebel *et al.*, 2019, 2020) despite repeated shifts back to bee pollination. Following the methods in Kriebel *et al.* (2020), we used PGLS and OUwie (Beaulieu *et al.*, 2012) analyses across the four groups of pollinators described earlier: bird-pollinated; bee-pollinated but derived from bird-pollinated ancestry (mainly in subgenus *Calosphace* but two in subgenus *Heterosphace*); bee-pollinated; and polymorphic. Evolutionary constraint on stigma morphology in subgenus *Calosphace*, despite pollinator switching, would be identified in two ways: (1) no significant differences in floral traits between bee- and bird-pollinated species in subgenus *Calosphace*; and (2) significant differences between the two bee-pollinated groups. We examined six cases of continuous character evolution with PC1 of stigma shape informed by the four pollinator regimes using BM and OU models implemented in OUwie v.1.53. We built seven classes of models (two BM and five OU processes) for each floral trait in which up to three parameters were estimated: θ (the phenotypic optimum), α (the strength of selection towards the optimum) and σ^2 (the evolutionary rate parameter). Models were compared using the

corrected AIC weights, which were summarized across the 11 trees. Since OUwie requires a priori mapping of pollinators on the phylogenetic trees, we performed stochastic character mapping on our 11 trees with all tips using the make.simmap function of the R library phytools implementing the all rates different model of discrete character evolution.

RESULTS

EVOLUTION OF *SALVIA* STIGMA SHAPE – STIGMAS OF NW HUMMINGBIRD- AND NW BEE-POLLINATED SPECIES ARE SIMILAR AND DISTINCT FROM OW SPECIES

A total of 567 outlines from 410 species (mean = 1.38; SD = 0.68) were included in the eFa of the stigma (Supporting Information, Fig. S1). Although we were generally not able to include multiple samples per species, all clades of *Salvia* were included. Furthermore, previous studies, the taxonomic literature and our own observations suggest intraspecific stigmatic variation is not evident and thus unlikely to influence the macroevolutionary patterns tested here. In addition, a study including 100 *Salvia* spp. (c. 10% of the genus) found that stigma shape was the only reliable character to separate the genus into two groups, suggesting that the morphology in either group (longer upper or lower stigma lobe) was consistent across many species (El-Gazzar *et al.*, 1968).

The eFa included 32 harmonics, which achieved 99.9% of harmonic power. Principal components analysis (PCA) of harmonic coefficients explained 70.9% of the variation (PC1 = 52.8%; PC2 = 18.1%). PC1 explains variation from a bilobed stigma with a longer and straight lower lobe, to a strongly asymmetric stigma with a much longer and curved upper lobe (Supporting Information, Fig. S2). PC2 is related to the orientation of the stigma lobes (especially the upper one) from erect to curved backwards. The match between 410 species with stigma shape data and the original 528 tip phylogenetic tree resulted in a reduced tree with 283 (69% of 410) tips. PC1 showed significant phylogenetic signal across this tree with a lambda = 0.84 ($P < 0.0001$) and $K = 0.69$ ($P = 0.001$). However, PC2 had no or little phylogenetic signal with a lambda = 0.000068 ($P = 1$) and $K = 0.12$ ($P = 0.053$). As PC1 both explains a significant amount of the variation in stigma morphology and is related to the hypotheses concerning stigma lobing, whereas PC2 does not, all subsequent analyses focus on PC1.

Of the 410 species in the morphometric data set, 402 were from *Salvia* and corresponded to 246 NW and 156 OW taxa. Scores for the outgroup were used in the shift detection analyses to allow for the possibility of shifts in the common ancestor of *Salvia*.

A morphospace showing the position of the outgroup which has a morphology similar to that of OW species is provided in the [Supporting Information \(Fig. S3\)](#). NW species occupy more than twice the area of morphospace relative to OW species (0.43 vs. 0.17 PC units) ([Table S1](#); [Fig. 2A](#)). Most OW species are embedded in one area of the NW morphospace, and

thus OW species exhibit an almost complete lack of unique stigma morphologies except for a few species with long and curved lower stigma lobes. Stigmas that are asymmetric with a longer upper lobe are lacking in the OW, whereas they are the most common but not the only type in the NW ([Fig. 2A](#)). The approximate randomization test comparing the mean shape of

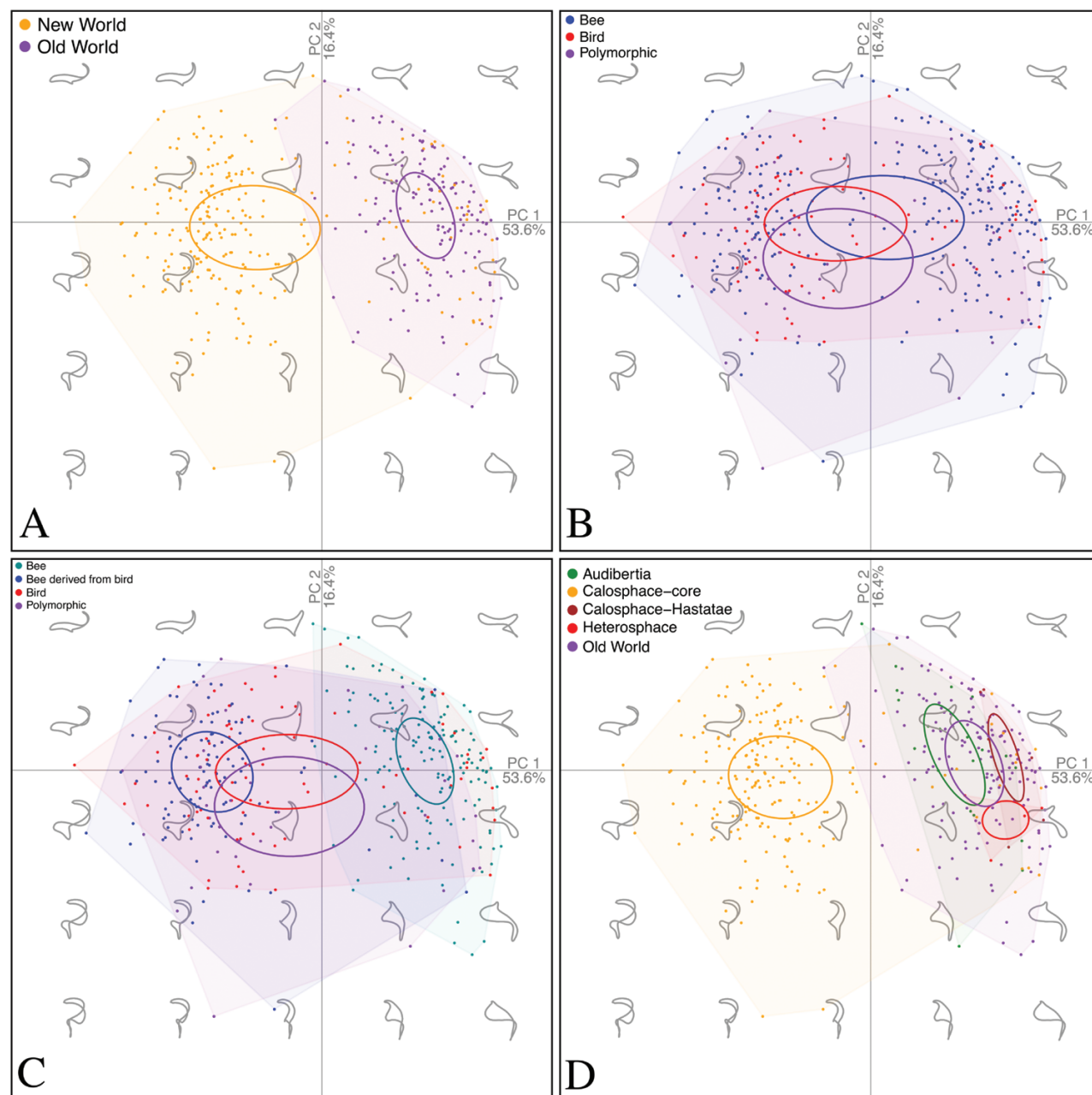


Figure 2. Morphospace variation of *Salvia* stigmas from elliptic Fourier analyses. Species are grouped in four ways: A, biogeographical area; B, pollinator (bee, bird, polymorphic); C, pollinator with bee derived from bird ancestry separated from bee; D, biogeographical area with New World separated by subclade. Percentages of the total variation explained by PC1 and PC2 for each of the three shape analyses are provided. Shaded polygons represent total morphospace occupied and ellipses the 50% confidence area for each area or pollinator.

the stigma by geographical distribution rejected the null hypothesis in favour of a difference in shape between NW and OW species for PC1 ($P < 0.001$; Table S2). PGLS comparing stigma shape on the reduced dataset matching the phylogeny and removing the outgroup for a total of 277 species between the OW ($N = 151$) and NW ($N = 126$) distribution, however, was not significant (mean $P = 0.44$, $SD = 0.14$ across trees; Table S2). Sensitivity analysis suggested that subgenus *Heterosphace* was probably influencing the model (Table S3), but its removal did not change the non-significant results in stigma shape by distribution.

The final pollinator tally for the 402 *Salvia* spp. in the morphometric dataset included 275 bee-pollinated, 87 bird-pollinated and 40 coded as polymorphic with potentially shared bee and bird pollinators. The bee-pollinated species can be further divided into 161 ancestrally bee-pollinated and 114 bee-derived-from-bird-pollinated. Of the 528 species in the phylogenetic trees, 519 are *Salvia* and are divided into 381 bee-pollinated, 103 bird-pollinated and 35 polymorphic. The bee-pollinated species can further be divided into 235 ancestrally bee-pollinated and 146 bee-derived-from-bird-pollinated. Of these, 106 have empirical observations on their pollinators (bee = 70; bird = 35; polymorphic = 1; Table S4). This includes species from across the geographical range of *Salvia* (NW bee = 10; NW bee from bird = 18; NW bird = 33; OW bee = 42; OW bird = 2). Of the 528 species in the phylogenetic trees, 128 have empirical observations on their pollinators (bee = 80; bird = 47; polymorphic = 1; Table S4), with 99 species shared with the morphometric dataset and showing broader representation of NW species (NW bee = 9; NW bee from bird = 30; NW bird = 45; OW bee = 41; OW bird = 2). The remaining NW species were coded for pollinator using an exhaustive list available for NW taxa (Wester & Claßen-Bockhoff 2006a, 2007, 2011) or if described after these studies based on similarity to species already coded. In the case of OW species, species without pollinator information were scored based on similarities to studied species (see Kriebel *et al.*, 2020 and Table S4 for additional references).

Broad overlap in area of morphospace is evident among pollinator types (Fig. 2B; Supporting Information, Fig. S3). However, the number of bird-pollinated species with symmetric or longer lower stigma lobes is small relative to bee-pollinated species, and most bird-pollinated species exhibit longer upper lobed stigmas. The approximate randomization test (Table S1) detected a significant difference between the morphology of bird- and bee-pollinated species for PC1 ($P < 0.001$), as did the PGLS (mean $P < 0.001$, $SD < 0.001$ across trees; Table S2). Sensitivity analysis found that subgenus *Heterosphace* was influencing the model significantly even after accounting for clade

size (Table S3), but its removal did not change the significant results in stigma shape by pollinator.

When bee-pollinated species are further divided into bees derived from bird-pollinated ancestors (most in subgenus *Calosphace*) vs. all other NW and OW bee-pollinated species, a strong separation in stigma morphology is seen between the two bee groups (Table S1; Fig. 2C). In support of the hypothesis that the stigmas of bee species derived from a hummingbird-pollinated ancestor would be similar to stigmas of related bird-pollinated species, these two pollinator groups (along with the NW polymorphic group) exhibit strongly overlapping mean confidence ellipses. Approximate randomization comparing the stigma morphology of bee-pollinated species derived from bird-pollinated species to the rest showed a significant difference between these taxa and the rest of the groups for the focal variable of PC1 (Table S2). PGLS analyses (Supporting Information, Table S2) also showed significant differences in stigma morphology between bee-derived-from-bird-pollinated species and the other three groups for PC1, with bee-derived-from-bird-pollinated species most similar in stigma morphology to bird-pollinated (and polymorphic) species (Fig. 2C). Sensitivity analysis found that subgenera *Audibertia* and *Heterosphace* influenced the model significantly even after accounting for clade size (Table S3), but their removal did not change the significantly different results between bee-derived-from-bird and the other groups.

SHIFT DETECTION ANALYSIS OF STIGMA SHAPE: EVOLUTION TO STRONGLY ASYMMETRIC STIGMAS

Shift detection analysis with 1100 of PC1 of stigma shape on the 283-tip MCC tree generated a best shift configuration with six significant shifts (Fig. 3; Table S5). The first shift was detected inside the NW subgenus *Calosphace* on the edge leading to the core-*Calosphace* (including all *Calosphace* except the *Hastatae* clade and *S. axillaris* and equal to Clade E *sensu* Walker & Sytsma, 2007). This shift found across all 11 trees (Table S5) involved a change from stigmas with equal lobes to stigmas with upper lobes being much longer and curved and a shortening of the lower lobe (Fig. 1). The remaining five shifts of two separate convergent regimes occurred in core *Calosphace*, and all involved shifts back to stigmas with longer lower lobe as seen in the background condition. The ten grove trees showed similar numbers and species compositions of shift reversals in core-*Calosphace* (Table S5).

The adaptation rate α ranged from 22.6 to 5635 across trees (mean = 847.5, $SD = 1674$), with six trees showing values of < 100 and one outlier > 1500 . The variance rate σ^2 ranged from 0.4 to 91.6 (mean = 13.8, $SD = 27.2$), with six trees showing values < 1.5 and

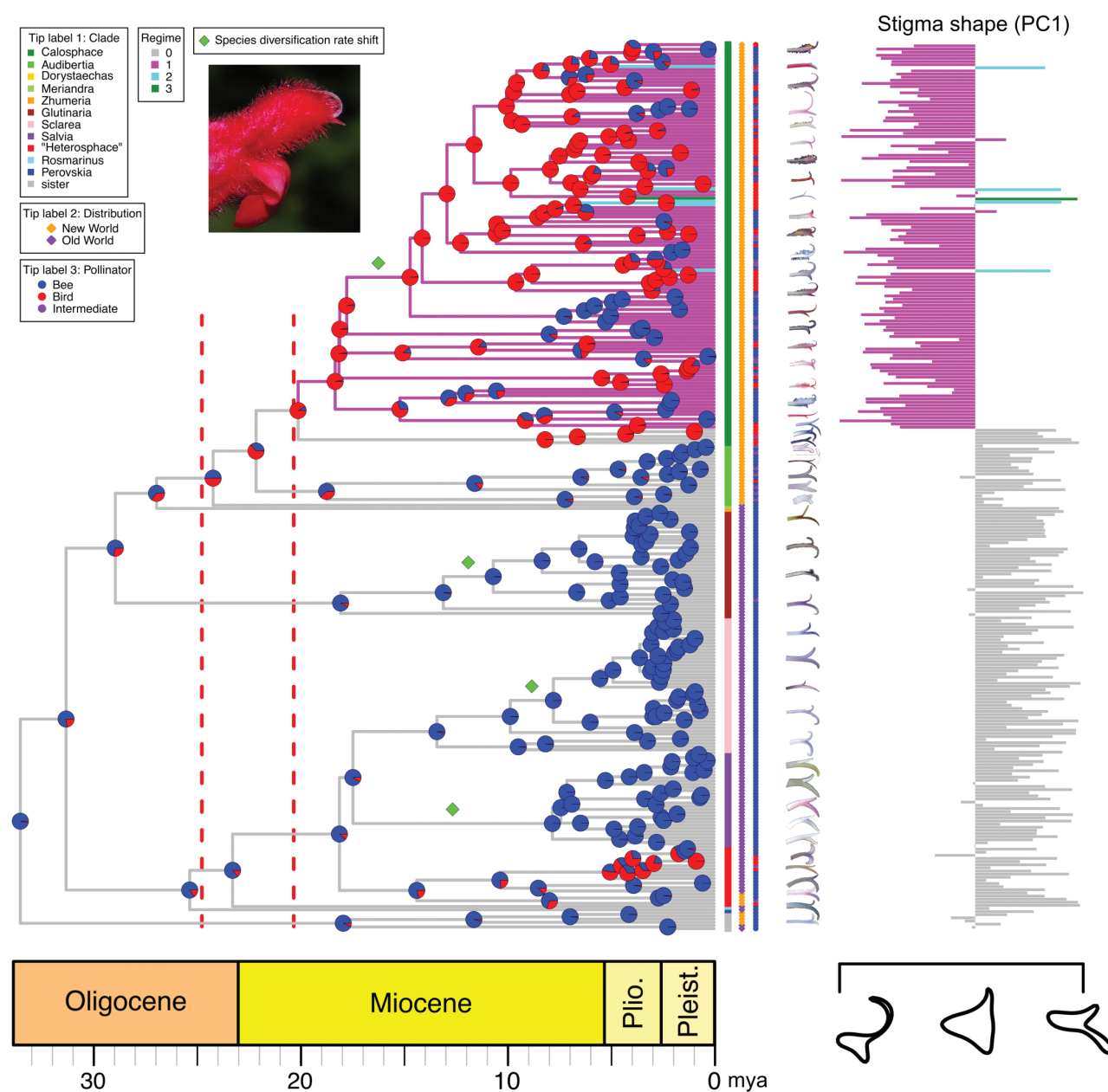


Figure 3. Univariate shift detection analysis with 11ou using PC1 from elliptic Fourier analysis of stigma shape in *Salvia* and close relatives. The left column depicts the phylogenetic tree with 263 tips based on the chronogram from Kriebel *et al.* (2019) with species dropped if not sampled. The four shifts in species diversification in *Salvia* from Kriebel *et al.* (2019) are indicated by green diamonds. Node pies represent the probability of each pollinator at each node. Tip labels (left to right) provide subgeneric placement and scoring for biogeographical area and pollinator. The right column depicts the score of PC1 for each tip (as a bar from the mean score) for stigma shape. Below the barplot a cartoon illustrates the mean (with its position indicated with a black dashed line) and the extreme shapes in PC1. Coloured PC1 lines (and the correspondent clade edges on the phylogenetic trees) represent separate regimes from the best shift configuration under the pBIC model selection criterion. Red dashed lines indicate ages of the stem and crown radiation of hummingbirds (McGuire *et al.*, 2014). The mouth of the corolla of hummingbird-pollinated *Salvia oxyphora* is shown to illustrate the curved stigma curving around the upper lip of the flower.

only one > 23 (the same outlier in the alpha values). With the alpha values we computed the phylogenetic half-life $[\ln(2)/\alpha]$, i.e. the time it takes the trait to reach half the distance to the optimum trait value. Half-lives were small, ranging from 0.03 to 0.0001 (mean = 0.008, SD=0.009). Small phylogenetic half-lives suggest rapid adaptation to new regimes, and low σ^2 values indicate little variation within regimes relative to the divergence between regimes (Uyeda & Harmon, 2014). This suggests that changes from a longer lower to a longer upper stigma lobe or vice versa in taxa that reverted to a longer lower stigma lobe in subgenus *Calosphace* occurred quickly, and that these morphologies are consistent within each group (mostly either longer lower or longer upper stigma lobe).

ANCESTRAL STATE RECONSTRUCTIONS: TRANSITIONS
TO STIGMATIC BRUSH AND LONG UPPER LOBES
OCCUR SIMULTANEOUSLY AT CORE-*CALOSPSPACE*
IMMEDIATELY FOLLOWING THE SHIFT TO
HUMMINGBIRD POLLINATION

Ancestral state reconstruction of pollinator type was conducted on the reduced 283-tip phylogenetic tree that matched species scored for stigma shape. The all rates different model of discrete trait evolution was used as the best-fitting model. The results presented here in inferring bee pollination as ancestral in *Salvia* with high probability (Fig. 3) are similar to those from recent studies that used trees with all or subsets of 528 species (Kriebel *et al.*, 2019, 2020). The main shift to bird pollination (hummingbirds) occurred with high probability on the MCC and ten grove trees at the crown of the NW subgenus *Calosphace* c. 20 Mya. This shift was subsequently followed by numerous reversals back to bee pollination. The few shifts to hummingbird pollination in subgenera *Audibertia* and *Heterosphace* occurred < 5 Mya.

Reconstruction of the stigmatic brush trait on the large tree of 528 tips included 185 species with a brush (only in subgenus *Calosphace*) and 343 species without a brush (some subgenus *Calosphace* and all other subgenera), and resulted in an ancestral state for the common ancestor of *Salvia* and close relatives as having no stigmatic brush (Fig. 4; Supporting Information, Fig. S5). The first origin of the stigmatic brush is found at the crown of core-*Calosphace* (18 Mya) one node after the major switch to hummingbird pollination occurred. The MCC tree showed 41 losses of the brush (37–42 across all grove trees) and two reversals back to the brush (one or two across all grove trees), all these transitions occurring in subgenus *Calosphace* (Fig. 4). Reconstruction of the stigma lobing trait included 241 species with longer upper lobe (only in subgenus *Calosphace*) and 287 species with longer lower lobe (some subgenus *Calosphace* and all other subgenera)

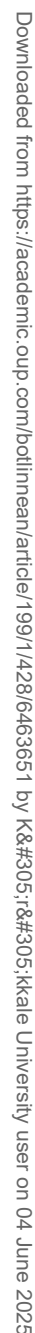
that is the ancestral state (Figs 4, S5). As with the evolution of the stigmatic brush, the first transition to a longer upper stigma lobe is at the crown of core-*Calosphace* just after the switch to hummingbird pollination. In core-*Calosphace*, the MCC tree (Fig. 4) showed 14 transitions back to lower lobe longer (12–18 across all grove trees) and three subsequent reversals back to upper lobe longer (one to seven across all grove trees).

POLLINATOR, STIGMATIC BRUSH AND STIGMA LOBING
TRAITS EVOLVE IN A CORRELATED AND CONTINGENT
FASHION ACROSS *SALVIA*

BayesTraits showed significant differences in transition rates between the two states: fewer transitions to bird pollination ($P < 0.001$), to stigmatic brush ($P < 0.001$) and to longer upper stigmatic lobes ($P = 0.002$), relative to more transitions in the reverse. All three traits evolve together in a significantly correlated fashion across *Salvia* and across subgenera *Calosphace* + *Audibertia* (Table 1) with the root states identified as bee pollination/longer lower stigma lobe (Fig. 5A), bee pollination/no stigmatic brush (Fig. 5B) and longer lower stigma lobe/no stigmatic brush (Fig. 5C); these are in agreement with ancestral state reconstructions (Fig. 4). The few shifts to bird pollination overall and with only one shift in subgenera *Calosphace* + *Audibertia* (Fig. 4) precluded effective contingent analyses involving bird pollination. However, the evolution of both the longer upper stigmatic lobes (Fig. 5A; q_{34}) and stigmatic brush (Fig. 5B; q_{34}) in a hummingbird pollination background in subgenus *Calosphace* is clear (Fig. 5).

The low rates of transition from the root to either of the derived states of the brush (Fig. 5C; q_{12}) or the longer upper stigma lobe (Fig. 5C; q_{13}) may reflect a limitation of BayesTraits. The program does not allow simultaneous evolution of two characters (i.e. diagonal transition from root to bottom corner, Fig. 5), but this joint evolution is implied in Figure 4. Subsequent transitions of these two stigmatic features in core-*Calosphace* are significantly contingent (Table 1). Evolution of longer upper stigmatic lobes only occurs in brush clades (Fig. 5C; q_{24}). Likewise, the brush primarily evolves in longer upper lobe clades (Fig. 5C; q_{34}). The correlative evolution of these two characters is pronounced considering that of the 185 species with a stigmatic brush (Fig. 5B, numbers on right corners), nearly all (99%) also possess longer upper stigma lobes (183) vs. longer lower lobes (two). Of the 242 species with a longer upper stigma lobe (Fig. 5C, numbers on bottom corners), 76% possess the stigma brush (183 vs. 59).

The repeated shifts back to bee pollination in subgenus *Calosphace* appear to be selectively neutral with respect to stigmatic lobing. Transitions back to



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bee pollination from clades with either longer lower or longer upper stigma lobes are not significantly different (Table 1; Fig. 5A). However, shifts back to bee pollination are significantly enhanced in clades possessing the stigma brush relative to no brush (Table 1; Fig. 5B). Bee pollination evolving from hummingbird-pollinated clades is three times more likely if the stigma brush is already present (Fig. 5B; q_{42} vs. q_{31}).

EVOLUTIONARY CONSTRAINT IN STIGMA MORPHOLOGY OF NW *SALVIA* SUBGENUS *CALOSPHERE*

The OUwie results also support the hypothesis that evolutionary constraint occurred in the diversification of stigma shape in subgenus *Calosphere* despite repeated shifts to bee pollination following the early transition to hummingbird pollination. Of the seven models examined, OUMA (OU model that assumes different state means as well as multiple α values per selective regime) and OUMVA (OU model that assumes different state means as well as multiple α and σ^2 values per selective regime) resulted in convergence problems and were thus excluded. Comparing the remaining five models resulted in the OUMV model as the best-fitting model (Fig. 6A), an OU model in which the stigma trait could have different phenotypic optimum (θ) and evolutionary rate parameter (σ^2) values.

The mean α value across trees was 1.43 (SD = 3.02) and inflated by the results of one tree that had an alpha of 10.5. The phenotypic optima for stigmas were more similar from bird-pollinated species and bee-derived-from-bird-pollinated species, and the phenotypic optimum from other bee-pollinated species was the most different (Fig. 6B). The mean σ^2 by pollinator was 0.02 (SD = 0.04) for bee, 0.04 (SD = 0.09) for bee from bird, 0.12 (SD = 0.25) for bird, and 0.09 (SD = 0.24) for polymorphic.

Regime shift with combined stigma, style, corolla and anther connective shape data occurs at the crown of core-*Calosphere*

Finally, we asked where regime shifts in floral shape occur in *Salvia* based on the holistic approach of combining stigma shape with previously obtained shape data for style, corolla and anther connective. Two significant shifts are detected (Fig. 7). The first is at the crown of the OW subgenus *Sclarea* (Moench) Benth., a node previously identified with shifts to strongly bilobed corollas and curved styles in response to specialized bee pollination (Kriebel *et al.*, 2020). The second significant shift is at the core-*Calosphere*, a node previously shown to be the edge with a change in corolla shape, a significant shift in anther connective and near the edge with a significant shift in style shape (Kriebel *et al.*, 2020).

Table 1. Likelihood ratio values for tests of correlated (4DF) and contingent (1DF) evolution of pollinator type (bee/bird), absence or presence of stigmatic brush, and stigmatic lobing (longer lower/upper lobe) within *Salvia* and within subgenera *Calosphere* + *Audibertia* using BayesTraits (values are separated by '/' for the two analyses). The number of shifts from bee to bird are too few to allow significant contingent analyses involving bird pollination See Figure 5 for details of tests for the *Salvia*-wide analyses

Test	Pollinator: stigma lobing (Fig. 5A)	Pollinator: brush (Fig. 5B)	Stigma lobing: brush (Fig. 5C)
Likelihood ratio of dependent vs. independent models (4DF)	Traits correlated: 14.28**/10.18*	Traits correlated: 18.60***/13.70**	Traits correlated: 51.45***/40.50***
Shifts to bee pollination occurs equally in longer lower and upper stigma lobe clades ($q_{31} = q_{42}$) (1DF)	Accepted: 0.24/0.59		
Shifts to bee pollination occurs equally in clades with and without stigma brush ($q_{31} = q_{42}$) (1DF)		Rejected: 5.95*/3.97* Shifts occur more in clades with stigma brush	
Shifts to stigma brushes occurs equally in longer lower and upper stigma lobe clades ($q_{12} = q_{34}$) (1DF)			Rejected: 10.6**/6.09* Shifts occur more in clades with longer upper stigma lobes
Shifts to long upper stigma lobes occurs equally in clades with and without stigma brush ($q_{13} = q_{24}$) (1DF)			Rejected: 4.21*/40.5*** Shifts occur more in clades with stigma brush

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

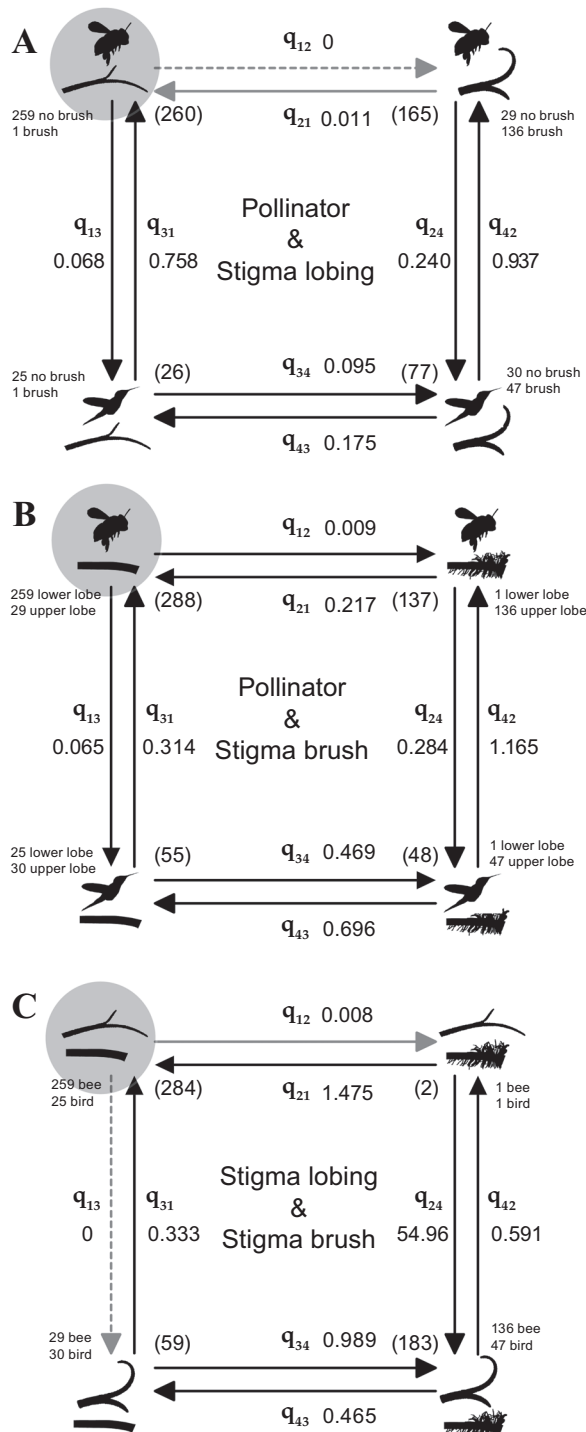


Figure 5. Correlative evolution of hummingbird pollination, longer upper stigma lobing and stigmatic brush across *Salvia* using BayesTraits. Pairwise tests include: A, bee/bird pollination and lower/upper longer stigma lobes; B, bee/bird pollination and absence/presence of brush; and C, lower/upper longer stigma lobes and absence/presence of brush. The eight rates of transitions (q) are provided for each. Transitions with $q = 0$ are dashed grey arrows;

DISCUSSION

The Neotropics are the setting of a diverse and complex geology, home to a great diversity of flowering plant species, the background for major radiations of hummingbirds (McGuire *et al.*, 2014) and bumble bees (Hines, 2008) and marked by intricate pollinator–floral interactions (Gentry, 1982; Jaramillo, Rueda & Mora, 2006; Antonelli *et al.*, 2009; Rull, 2011). The role of pollination ecology in the origin of such floral diversity is a fundamental issue in evolutionary biology (Mayer *et al.*, 2011) spurred by the demonstration of Sprengel (1793) that insects transport pollen to stigmas and the pioneering work on the origin of pollinator-driven floral diversity by Darwin (1862, 1876). The emerging paradigm focusing subsequent research (e.g. Stebbins, 1970; Mitchell *et al.*, 2009) has been that pollinators exert selective pressures on flowering plants and their floral traits and, in turn, that plants influence the evolution of animal pollinators. Phylogenetic comparative methods have increasingly been used to examine floral evolution, especially of the corolla, in the context of pollinators (e.g. Ibañez *et al.*, 2019) or shape (e.g. Lagomarsino *et al.*, 2017; Serrano-Serrano *et al.*, 2017; Joly *et al.*, 2018; Smith & Kriebel, 2018; Kriebel *et al.*, 2020).

The flower is, however, a complex suite of functionally and developmentally related traits, and natural selection can act on multiple traits simultaneously (Wessinger & Hileman, 2016; Bastide *et al.*, 2018). The occurrence of developmental drive (or bias, or constraint; Maynard Smith *et al.*, 1985) may arise if selection favours evolutionary change in one or a suite of characters while other related characters are selectively neutral. An earlier phylogenetic comparative study in *Salvia* documented pollinator-driven selection involving various sets of characters (corolla, anther connective and style) in specific clades and at different times and places (Kriebel *et al.*, 2020). We extend these findings in *Salvia* by demonstrating here the correlative and contingent nature of the evolution of two stigmatic traits to the rise of hummingbird pollination in the NW subgenus *Calospatha*: formation of a longer upper stigma lobe and a stigmatic brush. This study is the first to morphometrically quantify the shape of stigmas across a large clade of flowering plants and analyse stigma morphometric and discrete data in the context

transitions with $q > 0$, but not significantly so, are grey arrows. Numbers of species occupying each corner are given in parentheses, and these species are further divided by the state they possess of the third character not assessed for each test. For example, 260 species are bee-pollinated and have longer lower stigma lobes (upper left corner in A), and all but one of these species have no stigmatic brush.

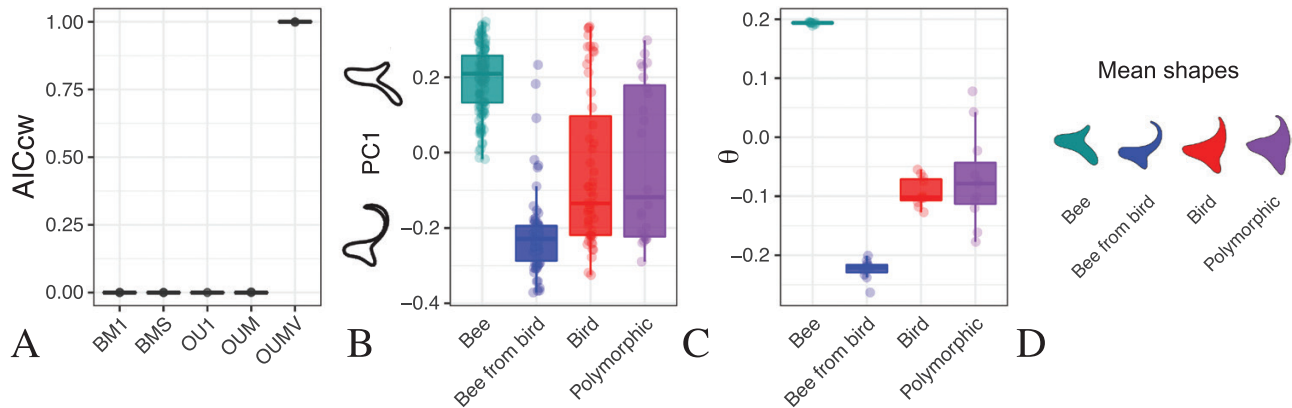


Figure 6. Evolutionary constraint on *Salvia* stigma morphology in bee-pollinated species derived from hummingbird ancestry depicted by OUwie analyses. Boxplots showing: A, the supported OUMV model across all trees based on corrected Akaike information criterion weights; B, PC1 scores of stigma shape of species by pollinator group; C, similarity of evolutionary optima (θ) of bee-pollinated species derived from bird ancestry and their hummingbird-pollinated relatives and dissimilarity of θ between the two bee-pollinated groups. D, mean stigma shape by pollinator group.

of specific hypotheses using phylogenetic comparative methods. As this study builds on previous analyses of corolla, anther connective and style shape evolution, it demonstrates the power and importance of dissecting flowers into separate components in morphometric analyses and using these data in phylogenetic comparative methods.

ASYMMETRIC STIGMAS WITH A LONG UPPER STIGMATIC LOBE AND THE STIGMATIC BRUSH EVOLVED EARLY IN THE MEXICAN AND CENTRAL AND SOUTH AMERICAN RADIATION OF *SALVIA*

The shift in stigmatic traits to longer upper lobes and possession of a brush occurred within the NW subgenus *Calosphace* (Figs 3, 4). Morphometric analysis of stigma shape that includes about half of the species of *Salvia* supports the suggestion of El-Gazzar *et al.* (1968) that these distinctive stigma traits are restricted to Central and South American species. With our higher number of species sampled, we expand the known distribution of the clade with upper stigma lobes and sub-stigmatic brushes going mainly from Mexico, through Central America to South America with *c.* 12 species in North America north of Mexico. The morphometric data support the hypothesis that asymmetric stigmas with a longer upper stigma lobe are restricted to subgenus *Calosphace*. Our results show that the stigma brush is also restricted to subgenus *Calosphace* (185 out of 266 sampled in the 528-tip phylogenetic trees, with ten species scored as polymorphic). Both the shift detection analyses (Fig. 3; Supporting Information, Fig. S4) and ancestral state reconstructions (Figs 4, S5) indicate that the evolution of these two stigmatic traits occurred just within the crown of subgenus *Calosphace*

(defined here as core-*Calosphace* and including all species in the subgenus except the *Hastatae* clade and *S. axillaris*) and excludes species of the small hastate-*Calosphace* clade from Mexico, Central America and northern South America.

Once derived, both stigmatic traits show more complex evolutionary patterns in subgenus *Calosphace*. Shift detection analysis indicated five shifts of two separate convergent regimes back to stigmas with symmetric or asymmetric lobes with a longer lower lobe. These reversals all occurred in hummingbird-pollinated species which exhibit exerted anthers and styles (Supporting Information, Fig. S6; Wester & Claßen-Bockhoff, 2006a, 2007, 2011). Stigmatic brushes were lost multiple times and re-evolved twice. However, the evolution of these two traits is correlated and contingent to some degree on switches to hummingbird and bee pollination.

HUMMINGBIRD POLLINATION GUIDED THE CORRELATED EVOLUTION OF A SUITE OF FLORAL CHARACTERS: STIGMA LOBING, STIGMATIC BRUSH, COROLLA, ANTHER CONNECTIVE AND STYLE

Significantly, the branch leading to the largely Neotropical NW core-*Calosphace* that exhibits both the shift in stigma lobing and origin of the stigmatic brush matches the placement of two floral shape shifts previously identified in *Salvia* (Kriebel *et al.*, 2020) involving the corolla and anther connective and is near the location of the style shape shift (Fig. 7). This suite of traits arose rapidly after the inferred transition from bee to hummingbird pollination at the crown of subgenus *Calosphace*. The last three traits and the unique staminal lever with fusion of the anther connective

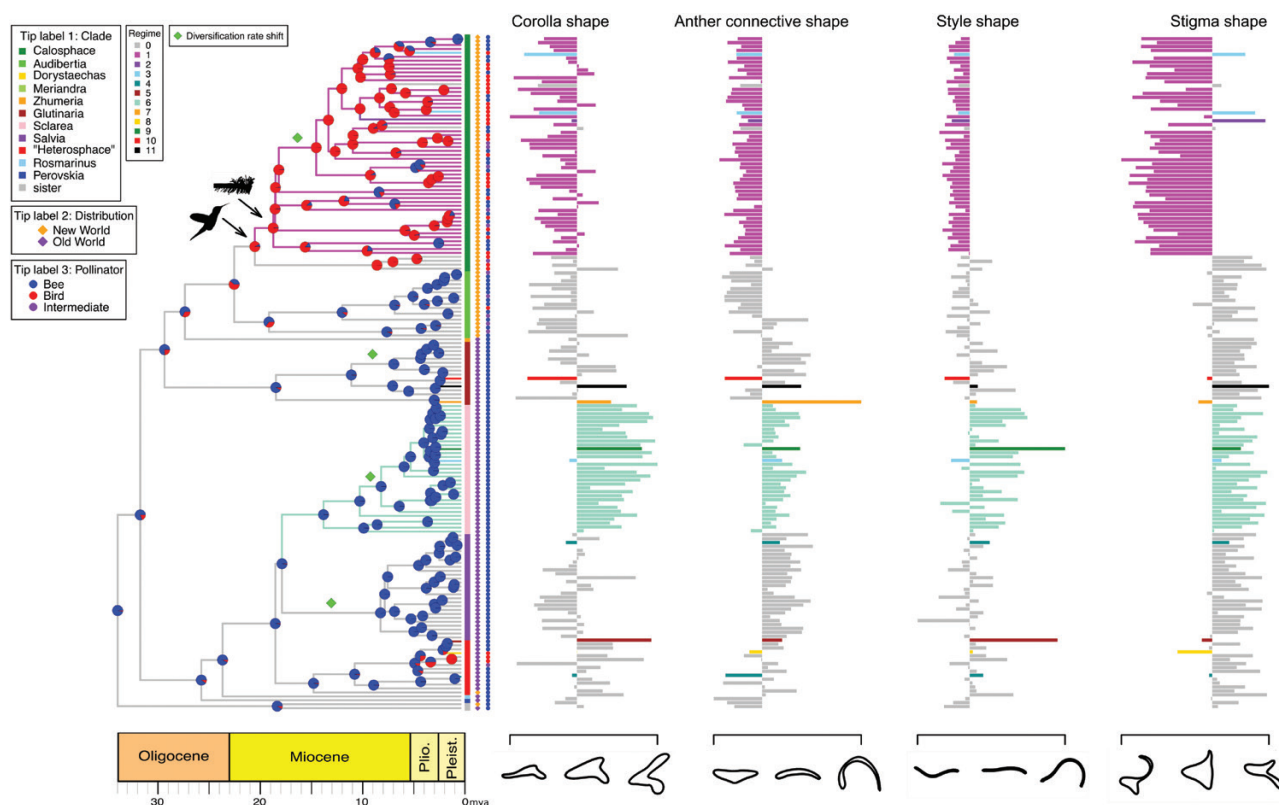


Figure 7. Multivariate l10u analysis across *Salvia* and close relatives combining morphometric stigma shape data with corolla tube, anther connective and style shape data from Kriebel *et al.* (2020). PC1 of each of the four traits was evaluated on a reduced 172-tip tree containing only species with all four data points present. Other details are as in Figure 3.

arms for most of their length (Walker & Sytsma, 2007) are adaptations for hummingbird pollination (Kriebel *et al.*, 2020, and references therein). Thus, despite repeated transitions back to bee pollination in core-*Calosphace*, the strong morphological differences of these traits maintained between OW bee flowers and NW bee and hummingbird flowers indicate a legacy of evolutionary constraints imposed by the hummingbird pollination shift 20 Mya in the Neotropics. Now we show that a larger suite of characters of both male and female function (corolla shape, anther connective shape, staminal lever, style shape, stigma lobing, stigma brush) arose together and exhibit correlated evolution guided by hummingbirds. The view of Wester & Claßen-Bockhoff (2007) that ‘bird pollinated species of *Salvia* confirm heterobathy’ (i.e. derived characters did not originate all at the same time but successively one after the other) needs to be tempered with the acknowledgement that this ostensibly was not the case early in the radiation of NW bird-pollinated *Salvia*.

It is notable that the small hastate-*Calosphace* clade (15 species from Mexico and Central/South America

exhibiting both hummingbird and bee pollination), sister to the core-*Calosphace*, lacks these floral innovations even though it also diversified after the shift to hummingbird pollination 20 Mya (Figs 3, 4). The selective advantage of these floral innovations in core-*Calosphace* appears tied to the significant rate shift in speciation centred in Mexico soon after (17.8–14.1 Mya), the oldest of all four rate shifts in *Salvia* (Kriebel *et al.*, 2019). It thus appears that hummingbirds guided the floral evolution involving at least five linked floral features in core-*Calosphace* over this time span, but they had little impact on the floral diversity or speciation rate of hastate-*Calosphace* during the same time span.

ADAPTIVE AND FUNCTIONAL SIGNIFICANCE OF THE LONGER UPPER STIGMATIC LOBE AND BRUSH

Although we have demonstrated a strong correlation between the evolution of the longer upper stigmatic lobe and stigmatic brush (and thus also to corolla, anther connective and style shape), the functions of the distinctive asymmetric stigma with longer upper lobes

and the presence of the brush seen in core-*Calosphace* have not been studied in *Salvia*. The BayesTraits analyses clearly demonstrate the importance of hummingbird pollination in initially driving these two stigmatic traits in core-*Calosphace*. Subsequent shifts back to bee pollination in the Neotropics may well have been facilitated by these innovations, particularly the stigmatic brush. The shifts to bee pollination in this clade are significantly correlated with the presence of the brush, whereas the longer upper stigmatic lobe appears to be selectively neutral with respect to these shifts to bee pollination. Our hypothesis is that both the longer upper stigma lobe and sub-stigmatic brush are innovations selectively advantageous in secondary pollen presentation (Howell *et al.*, 1993; Yeo, 1993) by ensuring greater precision in pollen placement and longer pollen presentation, especially with regard to hummingbird pollination, and at least the stigmatic brush is selectively advantageous to bee pollination.

The basis for this secondary pollen presentation hypothesis in *Salvia* is as follows. First, *Salvia* flowers are zygomorphic, provide a nectar reward and are protandrous, with anthesis occurring before stigma receptivity (Navarro, 1997) as in most Lamiaceae (89%, Bertin & Newman, 1993). Such specialized flowers with constant nectar production permit species to vary separation of male and female function in time (dichogamy) with selection operating on the synchrony of pollen discharge and pollen receptivity in different flowers (Lloyd & Webb, 1986; Bertin & Newman, 1993).

Second, both OW bee- and bird-pollinated *Salvia* spp. typically exhibit separation of male and female function in space (herkogamy), with exerted styles relative to the anthers (Supporting Information, Fig. S6; Wester & Claßen-Bockhoff 2006a, b; Celep *et al.*, 2020; Kriebel *et al.*, 2020). However, most species in NW core-*Calosphace* have a much reduced herkogamy with the stigma lobes near the corolla opening (Fig. S6; Kriebel *et al.*, 2020), and with the upper longer stigma lobe appearing to act as an anchor on the top of the upper corolla lobe or galea [Fig. S6; see fig. 3 in Wester & Claßen-Bockhoff (2007) and fig. 9 in Wester & Claßen-Bockhoff (2006a)]. Howell *et al.* (1993) argued that precision in pollen placement on pollinator and deposition on stigmas is maximally achieved through the reduction and even elimination of herkogamy within a flower. This enhanced precision in core-*Calosphace* is thus related to specific hummingbird (and later bee) pollination, reduced herkogamy and specialized upper stigma lobe function and may have been instrumental in its radiation (Caruso *et al.*, 2018). The much longer upper stigma lobe wrapped around the galea in this distinctive floral construction may have originally evolved as a mechanism to avoid intra-floral sexual interference (Lloyd & Webb, 1986; Webb & Lloyd, 1986; Barrett, 2002b) by keeping

the style in place when the lever moves down, thus allowing the smooth functioning of the lever. In this respect the upper lobe acquiring a new function would be an exaptation (*sensu* Gould & Vrba, 1982). Through time, it is possible some species retained this longer upper lobe because it is constrained and not easily lost unless other selective forces operate, such as perhaps interactions with other hummingbirds. The few species that have reverted to a longer lower lobe are hummingbird-pollinated but have evolved an exerted style that usually changes back to a longer lower lobe. Experimental work removing the upper stigma lobe and activating the lever may clarify if it indeed helps anchor the style.

Third, stigmatic brushes, which we demonstrate are correlated to the longer upper stigmatic lobe in *Salvia*, are common in SPP plants in which they function as pollen traps (as in other plants with anther trichomes; Hesse, Vogel & Halbritter, 2000) to prolong pollen presentation (Lavin & Delgado, 1990; Howell *et al.*, 1993; Ladd, 1994; Leins & Erbar, 2006; Aximoff & Freitas, 2010). In core-*Calosphace*, the anthers are frequently kept hidden inside the upper corolla galea and maintained at the level of the style brush. As the brush often contains pollen (Supporting Information, Fig. S6), it is likely that the long upper stigmatic lobe and its brush function synchronously to prolong pollen presentation similar in fashion to other SPP plant species. Presumably, this suite of features held within the corolla galea was strongly selected for in NW *Salvia* subgenus *Calosphace* as a unique pollen deposition and presentation mechanism initially for hummingbird pollination and maintained and used, at least in part (the stigmatic brush), following subsequent switches to bee pollination. Some bee-pollinated species in subgenus *Calosphace* have the sub-stigmatic brush restricted almost in its entirety to the lower surface (e.g. *Salvia farinacea* Benth.), and these brushes may also represent an exaptation where the brush that originally evolved for secondary pollen presentation (as in most angiosperms that have sub-stigmatic brushes) was secondarily co-opted to remove competing pollen from incoming bees. The stigma is placed in the pollinators' path prior to them entering the area between the galea and the lower lip where the brush is positioned. Thus, after they have touched the stigma and before they press the lever, the brush could remove foreign pollen from these bees as they move towards the tube orifice in search of nectar.

The shift to hummingbird pollination c. 20 Mya near the crown of the largely Neotropical NW *Salvia* subgenus *Calosphace* and soon after the radiation of hummingbirds (McGuire *et al.*, 2014) set in motion a series of drastic changes in corolla, anther connective and associated lever, style features (Kriebel *et al.*,

2020), and, as demonstrated here, in two key features of the stigma, the evolution of a longer upper-stigma lobe and the stigma brush. These correlated characters facilitated one of the most spectacular plant radiations in the Neotropics, a radiation which also exhibited repeated and successful transitions back to bee or mixed pollination strategies. These transitions back to bee pollination were undoubtedly influenced by the several radiations of Neotropical bumble bee lineages starting as early as 15 Mya (Hines, 2008). However, key questions remain unanswered in this group. How do these correlated characters function specifically in promoting hummingbird pollination? Did the evolution of the stigma brush and longer upper lobe, thought to be important in aiding outcrossing through secondary pollen presentation, intensify sexual selection in the clade? How was the evolution of this syndrome, after millions of years, exploited independently multiple times by bees? What local community, habitat or climatic factors moderate the selective pressures on visitation by hummingbirds or bees on different *Salvia* spp. and how did these factors impact the diversification of *Salvia*? Answering these questions will require a consideration of the evolutionary background in which these characters evolved 20 Mya, in the context of a shift to hummingbird pollination in *Salvia* subgenus *Calosphace*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Morphospace occupation and comparisons of *Salvia* stigmas by OW and NW geographical area (Test A), three pollinator groups (Test B) and four pollinator groups with NW bee-pollinated species derived from hummingbird-pollinated ancestry separated from other NW and OW bee-pollinated species (Test C). Area of morphospace is given in PC units that are only comparable within the PCA of the stigma. The percentage of morphospace area shared is relative to the total morphospace area occupied by both groups. Significant differences

in stigma shape when comparing either area or pollinator were evaluated by approximate randomization tests with 10 000 simulations. Letters in parenthesis correspond to group assignments for PC1 and PC2 resulting from pairwise approximate randomization tests with 10 000 simulations.

Table S2. Phylogenetic generalized least squares (PGLS) regression models comparing the morphology of *Salvia* stigmas represented by PC1 by OW and NW geographical area (Test A), three pollinator groups (Test B) and four pollinator groups with NW bee-pollinated species derived from hummingbird-pollinated ancestry separated from other NW and OW bee-pollinated species (Test C). Parameters are given as averages across 11 trees.

Table S3. Results of sensitivity analyses testing for the influence of the major clades of *Salvia* on model parameters of phylogenetic generalized least squares regression models. For these tests, major clades with more than five species were sequentially removed (*Audibertia*, *Calosphace*, *Glutinaria*, 'Heterosphace', *Salvia* and *Sclarea*) and model parameters were estimated and compared to the full model. To account for the size of the clade and discount that the influence is only due to clade size, a randomization test is conducted and if $P < 0.05$ then the influence is not only due to the size of the clade. DIFest. indicates the change in slope when removing a particular clade. The significance column shows the percentage of the trees with a significant result.

Table S4. List of studies reporting empirical observations of likely pollinators of sages. A few observations are reported here for the first time.

Table S5. Results from shift detection analysis of stigma shape as represented by PC1 in *Salvia* and outgroups with 11ou showing the taxa involved in shifts across the MCC and ten grove trees. Numbers assigned to taxa correspond to their resulting regime, and identical regime numbers in a tree signifies they converged to the same regime. A value of 0 means convergent evolution to the background regime of the tree. The number of regimes is not necessarily the same across analyses. All additional shifts besides the one in core *Calosphace* occurred in *Calosphace*.

Figure S1. A, stigma outlines of species from *Salvia* and close relatives obtained from floral scans or from the literature used in morphometric analyses ($N = 567$ representing 410 species). B–D, examples of stigmas with longer lower lobe (A), equal lobes (B) or longer upper lobe fitted with four landmarks.

Figure S2. Morphological variation of *Salvia* stigmas explained by each of the first two principal components from elliptic Fourier analyses. For each structure, the first row corresponds to the mean shape in the middle column $\pm$ the standard deviation of the variation explained by PC1 and the second row by PC2.

Figure S3. Morphospace of stigma shape with specimens grouped by pollinator as in Figure 2B but also showing the placement of the sister group, which includes species of *Lepechinia* and *Melissa*.

Figure S4. Univariate shift detection analysis with 11ou using PC1 from elliptic Fourier analysis of the stigma in *Salvia* and close relatives. Figure identical to Figure 3 but with species tips added.

Figure S5. Ancestral state reconstruction across *Salvia* of the stigmatic brush and stigma shape as a discrete variable. Figure identical to Figure 4 but with species tip added.

Figure S6. Examples of sexual interference strategies employed by *Salvia*. A, B, rotandry in *Salvia rosmarinus* with (A) male phase and (B) female phase. C, herkogamy in *S. somalensis*. D, pronounced herkogamy in *S. pratensis* (note strongly exerted style). E, F, heterostyly or reciprocal herkogamy in *S. yangii* depicting (E) thrum flower with exerted stamens and short style and (F) pin flower with exerted style and short stamens. G, approach herkogamy in *S. haenkei*. H, reduced approach herkogamy in *S. roemeriana*. I, corolla of *S. oxyphora* with the side of the corolla removed showing the anther connective, anther and stigma. J, same as in I above but with the anthers levered showing how pollen is deposited on the stigma brush.