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Phenotypic selection mosaic for flower length influenced by geographically varying hawkmoth pollinator proboscis length and abiotic environment

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Summary

- Biotic and abiotic context may affect the intensity of interspecific interactions and subsequently drive locally particular phenotypic selection patterns on interacting traits.
- We evaluated the geographical variation of matching traits of the brush-type flowers of *Caesalpinia gilliesii* and of the proboscis length of its guild of hawkmoth pollinators, as well as their relation with environmental variables. We assessed the geographical variation of interacting traits (style and filament vs. mean proboscis length of the guild of hawkmoths) across seven populations, and estimated phenotypic selection on the plant side.
- Interacting traits showed similar relationships with environmental variables. Phenotypic selection on the plant side was influenced by proboscis length and by environmental conditions. Mean proboscis length of the guild was shorter than previously recorded for the same study area thus probably shifting the selective optima of flower length. We observed two presumptive coevolutionary cold spots where one-sided negative directional selection is acting on style length. The lack of selection on the pollinator-side should be further confirmed.
- We provided joint evidence, mostly lacking, about the geographical variation of selective pressures on the plant side associated with both proboscis length and abiotic conditions. We suggest that recent environmental change may be shifting floral length optima.

Key words

abiotic environment, coadaptation, geographic range, nocturnal plant-pollinator interactions, phenotypic selection mosaic, trait-matching

1. Introduction

From a population perspective, coevolution of phenotypes, in its more elementary form, is the evolutionary process among interacting species pairs by which traits of one species cause evolutionary change in the interacting traits of another species. Change in the second species, in turn, causes evolutionary change in interacting traits of the first species. In an extended, more realistic form, reciprocal evolutionary change and reciprocal match of interacting traits is attained by multiple species causing evolutionary change in the same direction (Janzen, 1980; Althoff *et al.*, 2014). To disentangle whether coevolutionary processes are actually shaping species traits to mutual match, interspecific relationships and phenotypic selection patterns should be evaluated across the distribution ranges of the interacting species (Benkman *et al.*, 2001; Toju & Sota, 2006; Pauw *et al.*, 2009; Siepielski *et al.*, 2013). This is the approach derived from the Geographical Mosaic Theory of Coevolution (GMTC) which posits that a coevolutionary processes occurs because the following: first, interspecific interactions vary geographically, structuring the patterns of phenotypic selection such that reciprocal selection only acts in some populations, and, second, genetic changes are subjected to historical dynamics across the landscape due to gene flow, genetic drift and local extinction (Thompson, 1994, 2005).

The coevolution of mutually matching traits among species engaged in pollination interactions has been and still is a favorite study subject since the origin of the coevolution concept. Without using the term “coevolution” the idea was first approached by Darwin, (1859, 1863) and has been further elaborated up to the present (Janzen, 1980; Johnson *et al.*, 1997; Ehrlich & Raven, 2006; Zimmer & Emlen, 2013). When plant species are highly dependent on pollinators for their reproduction, pollinator-mediated selection is critical in shaping traits involved in pollen receipt and export and, reciprocally, when the fitness of pollinators is affected by their interaction with flowers, plant-mediated selection is critical in shaping the traits of pollinators (Armbruster *et al.*, 2009; Eklöf *et al.*, 2013; Sazatornil *et al.*, 2016; Lomáscolo *et al.*, 2019). Plants and pollinators evolve specialized phenotypes in the context of interactions which generally are asymmetric with one plant species being ecologically specialized, i.e. dependent on a narrow number of species of functionally equivalent pollinators (e.g. hummingbirds or hawkmoths). Conversely, each pollinator species is often an ecologically generalist, foraging from a wider range of species which may or not belong to the same plant guild, e.g. ornithophilous or sphingophilous (Ashworth *et al.*, 2004; Bascompte *et al.*, 2006). Matching traits will coevolve regardless of the degree of specialization of interacting partners because species bearing

specialized traits would profit from being able to widen their set of partner species to include those with the more specialized traits, in addition to the phenotypically less specialized ones (Sazatornil *et al.*, 2016; Lomáscolo *et al.*, 2019). At the same time, the geographical structure, given by the varying ecological contexts where interactions take place, is carried over to variation among local interaction networks and, subsequently, to variation in the strength and mode of selection on complementary traits across communities (Thompson, 2005; Anderson & Johnson, 2008; Pauw *et al.*, 2009).

The strength and mode of the interaction between plants and pollinators, and consequently the phenotypic selection on interacting traits, are influenced by the abiotic contexts. For instance, clinal and altitudinal variations of the volume and type of floral reward to pollinators, and of interacting trait-matching, as well as their changes with temperature and precipitation have been extensively reported (Anderson & Johnson, 2008; Pauw *et al.*, 2009; Nattero *et al.*, 2011; Cosacov *et al.*, 2014; Ferreiro *et al.*, 2015). Here we postulate that the relationship between floral traits and environmental variables inform us about the environmental feasibility of trait measures (see below). In conjunction with these factors, selection of reward-collecting appendages and complementary flower traits by ecological partners should result in close reciprocal trait-matching in each population where the magnitude of traits differs between populations (Johnson & Anderson, 2010). As expected by this principle, morphological co-variation of interacting traits across populations has been recorded for a number of plant-pollinator systems (Steiner & Whitehead, 1991; Moré *et al.*, 2006; Anderson & Johnson, 2008, 2009; Pauw *et al.*, 2009; Nattero *et al.*, 2011; Cosacov *et al.*, 2014) but differential selection of these traits across populations has seldom been tested (but see Caruso *et al.*, 2003; Gómez *et al.*, 2009; Emel *et al.*, 2017; Ferreiro *et al.*, 2017).

According to the GMTTC, selective pressures should change across the geographical range of interacting species, with “coevolutionary hot spots” where community partners select each other reciprocally, and “coevolutionary cold spots” where selection among community partners is either one-sided or absent (Anderson & Johnson, 2008; Pauw *et al.*, 2009; Ferreiro *et al.*, 2017; Soteras *et al.*, 2018). For the evolution of flower and pollinator proboscis lengths in a hot spot, positive directional evolution on both parties is expected (Nilson & Nilsson, 1988; Pauw *et al.*, 2009; Fig. 1a, on the hot spot area). Such a process implies a positive shifting of the local optimum, which will obviously find a viability limit, given by conditions other than reciprocal selection such as environmental feasibility of developing extremely long traits. When the shift of

the optimum reaches its environmental feasibility, then reciprocal stabilizing selection should be expected until phenotype and fitness variation is depleted and the hot spot turn into a cold spot (Fig. 1b, on the cold spot area). In a cold spot, if selection occurs at all, flower length could, for instance, be subjected to positive or negative directional one-sided selection to meet pollinator traits without affecting the selection of the complementary pollinator trait (Fig. 1c,d, on the cold spot area). A scenario of one-sided evolution could be created when processes other than co-evolution, such as local extinction of members from one side, set a new local adaptive optimum. This theoretical scenario (Fig. 1) represents the coevolutionary patterns expected by the sole influence of environment and interacting traits, not considering the effect of trait remixing. Gene flow among populations may maintain the overall pattern of hot and cold spots by adding new sources of variation. In addition, the scenario could change by the random effects of genetic drift, local extinction, recolonization of populations, and mutation (Thompson, 2005; Gomulkiewicz *et al.*, 2007).

For the GMTC perspective, it should be acknowledged that selection on traits is influenced, not only by interaction partners, but also by abiotic constraints which may condition selection regimes and, as said above, could set an upper limit for optima to shift. A recent meta-analysis has shown that selection regimes are significantly influenced by climate, independently of the functionality of the traits being selected (Siepielski *et al.*, 2017). For example, precipitation could eventually pose limits to the evolution of flower length if its development is resource limited or sensitive to water availability. Despite that some studies have addressed the relationships of phenotypic selection with abiotic (Siepielski *et al.*, 2017) and biotic variables (Anderson & Johnson, 2008; Pauw *et al.*, 2009; Ferreira *et al.*, 2017) across the geographical range of interacting species, to the best of our knowledge, none has evaluated the joint influence of both kind of variables on phenotypic selection (but see meta-analysis of Caruso *et al.*, 2019).

In the present study we aimed to evaluate whether geographical co-variation of flower-pollinator interacting traits and phenotypic selection of flower traits in the widespread hawkmoth pollinated plant *Caesalpinia gilliesii* is consistent with a coevolutionary process in an ecological geographical mosaic of interactions. We evaluated the geographical variation of floral phenotype in *C. gilliesii* and in the proboscis length of its guild of hawkmoth pollinators, as well as their relation to environmental variables such as latitude, elevation, temperature, and precipitation. In addition, we evaluated if there is a geographical pattern of phenotypic selection on the plant side, particularly of two functional interacting traits, filaments and style length. We studied seven

natural populations of *C. gilliesii* to answer the following questions: 1) If any, which is the importance of environmental variables in phenotypic variation? 2) Does phenotypic selection vary among populations? 3) Which is the relative importance of environmental variables and proboscis length in explaining phenotypic selection variation?

2. Materials and Methods

2.1. Plant species and study sites

Caesalpinia gilliesii (Wall. ex Hook.) Dietr. (Fabaceae) is a native andromonoecious shrub of up to 3m in height which grows in arid and semi-arid regions of Argentina, and is widely cultivated as ornamental plant (Parodi, 1987). This species does not set fruits by self-pollination, depending almost exclusively on long-tongued nocturnal hawkmoths to assure fertilization (Moré *et al.*, 2006). The brush-type fragrant flowers with exerted styles longer than filaments, are arranged in inflorescences and bloom sequentially at dusk (Cocucci *et al.*, 1992). Filaments and style length, as well as the composition of the guild of pollinators vary geographically (Moré *et al.*, 2006). In addition, these authors observed that where the species showed the longest styles, only the pollinator species bearing the longest proboscis occurred (Moré *et al.*, 2006).

We studied seven populations in Argentina spanning the geographical range of *C. gilliesii* which are located between 26.59° S and 36.91° S, and between 63.59° W and 67.51° W (Table S1). Surveys were carried out during the flowering season (from October to December) in three consecutive years (from 2015 to 2017).

2.2. Phenotypic traits and fitness metrics

In each population, 60 adult flowering individuals of *C. gilliesii* separated by at least two meters from each other were haphazardly chosen to measure phenotypic traits (Table S1). In each plant, we counted the number of inflorescences and open flowers, as indicators of resource status and attractants to pollinators. From each plant, three newly open flowers from different inflorescences were collected to measure style and filament length and flag petal area, as a metric of flower size and display. Scaled photographs of the flowers were taken using a Nikon Coolpix 5400 camera and measurements were taken from the digital images using the SigmaScan® Pro 5.0 Software.

Three inflorescences per plant were chosen to quantify number of seeds per fruits, which can be visualized directly in the field before seed dispersal by the explosive seed pods, and fruit set per inflorescence (number of developed fruits in relation to total number of flowers). Total number of flowers could be quantified since the flowers which do not persist leave an evident scar

on the inflorescence axis. In addition, from each plant we collected 10 stigmas of flowers that had opened the night before and had dehiscent anthers showing the complete pollen load. We counted the number of pollen grains of *C. gilliesii* deposited per stigma under a stereomicroscope (Leica MS5). Because pollen grains were not indicative of seed set (Pearson correlation $r=-0.025$, $P=0.613$) we did not include this fitness metric for further selection analysis. We estimated the following metrics of female fitness: number of seeds per fruit, number of fruits per inflorescence, number of seeds*number of fruits per inflorescence, and number of seeds*number of fruits per plant.

For the historical data-set of floral traits, we used style and filament lengths of five populations (Ampimpa: 7 individuals, Cuesta Blanca: 17 individuals, El Arenal: 6 individuals, El Carmen: 11 individuals, Luro; 28 individuals) sampled during the years 2000 to 2002 (Moré *et al.*, 2006, 2014).

2.3. Pollinators

The recorded guild of pollinators of *C. gilliesii* in western and central Argentina includes four long-tongued hawkmoth species: *Manduca bergi*, *M. diffissa*, *M. sexta* and *Lintneria maura* (Moré *et al.*, 2006). For the pooled data, proboscis length data range from 44 to 105 mm, long enough to touch anthers and stigma with their bodies while drinking nectar and not too long as to drink nectar without touching anthers and stigma. Hawkmoth species bearing proboscis too short or too long (e.g. *Callionima girsescens* or *M. rustica*, respectively) and diurnal visitors such as *Apis mellifera*, *Bombus pauloensis* and *Xylocopa* sp. have been occasionally observed carrying small amounts of pollen of *C. gilliesii* (Cocucci *et al.*, 1992; Moré *et al.*, 2006). However, these species rarely touch the stigmas and are, thus, regarded here more as nectar robbers than as actual pollinators.

To account for possible changes in the composition and proboscis length of the *C. gilliesii* hawkmoth pollinator guild during the past 10-17 years and, thus, for possible shifts in the plant's adaptive optima, we characterized the hawkmoth guild both from current and historical data sources. For the current data, hawkmoths were caught using one vertical sheet light trap illuminated by two 250 W mix light bulbs located at least once in each population, totaling 13 nights (4 h in Amaicha, 8 h in Famatina, 4 h in Valle Hermoso, 12 h in Manantiales, 16 h in La Calera, 4 h Rio Primero, 4 h in Luro). Each trapping lasted 4 h, from 20:00 to 24:00 h. Hawkmoths that settled on the sheets were immobilized with alcohol 70% and kept in entomological envelopes for further identification and analysis. From each individual, measurements of proboscis and wing

length were taken with a digital caliper to the nearest 0.1 mm. Pollen load of *C. gilliesii* was determined under a stereomicroscope Leica (MS5).

For the historical data-set, we used own records of 456 individuals collected during the years 2000 to 2005 along a wide geographic range encompassing the current study populations (Moré *et al.*, 2006, 2014). These individuals were collected with the same procedure as above.

2.4. Statistical analysis

2.4.1 Association of floral traits with environmental variables

Total variance of each phenotypic trait was partitioned into its hierarchical component, i.e. among populations (n=7), among individuals (n=60) and within individuals (n=3), using a mixed-effect nested model for each measured trait. Significant differences of each level were tested with restricted ML estimation (REML). These analyses were performed with the *lme4* package (Bates *et al.*, 2014) in R (R Core Team, 2019).

To determine whether and how much of the phenotypic differences among populations were explained by the geographic distance among them, we tested the association between both distance matrices using a permutation test based on Procrustes statistic (PROTEST). In a Procrustes analysis the pair of data matrices is compared by using a rotational-fit algorithm that minimizes the sum of the squared residuals between the two matrices (Jackson, 1995). The resulting goodness-of-fit statistic (m^2) is a measure of dissimilarity between the two configurations and varies between 0 and 1, with small values indicating low dissimilarity (Jackson, 1995). The significance of the congruence was determined with 10000 permutations. We expressed congruence between the two matrices as $(1-m^2) \times 100$ (Alarcón, 2010). These analyses were performed using the *vegan* package (Oksanen *et al.*, 2019) in R.

The phenotypic trait matrix was partitioned among environmental variables using distance-based redundancy analysis (db-RDA), with *capscale()* function from R package *vegan* (Legendre & Anderson, 1999). The following predictor variables were included: latitude, elevation, mean annual temperature, mean annual precipitation, mean temperature of the wettest quarter, mean temperature of the warmest quarter, temperature seasonality, mean precipitation of the wettest quarter, mean precipitation of the warmest quarter, and precipitation seasonality. Bioclimatic variables were obtained from the DRYAD database (Vega *et al.*, 2017a) from MERRAclim (Vega *et al.*, 2017b). All explanatory variables were standardized to zero mean and unit variance. Variation explained by environmental variables was determined using forward model choice on adjusted R^2 with 999 permutations using the *ordiR2step()* function. In this procedure, the variables

that best fit the data are sequentially selected and added to the final model. These analyses were performed using the *vegan* package in R. In order to evaluate variation of phenotypic traits in relation to the selected environmental factors (latitude, elevation, temperature seasonality, temperature of the wettest quarter, mean annual temperature, and mean annual precipitation), we fitted generalized linear mixed models including populations as random term. We assumed that these relationships estimate the mean environmental feasibility of the floral traits across the environmental range. Hence we could have an estimated local optimum for each trait.

2.4.2. Proboscis length relationships with environmental variables and flower length

We assessed the relationship of previous records of proboscis length and environmental variables by general linear models. We predicted proboscis length of the guild of hawkmoths of each population as a function of latitude and elevation using the previously recorded data of the guild of hawkmoths (n=456, Moré *et al.*, 2006, 2014). We determined the relationship of style and filament with predicted proboscis length.

2.4.3. Geographical variation of phenotypic selection

Opportunity for selection (I) acting on each population was estimated as the variance for four female fitness metrics: number of seeds per fruit, number of fruits per inflorescence, number of seeds*number of fruits per inflorescence, and number of seeds*number of fruits per plant (Brodie III *et al.*, 1995).

Phenotypic selection was estimated for both the pooled data set (Emel *et al.*, 2017) and separately for each population (n=60). Style length and filament length, were used as predicting variables and the above four fitness metrics as response variables. Since total number of fruits or flower scars were sometimes different among individuals, these were added as an offset in order to account for differences in replicates (Zuur *et al.*, 2009). To assess the magnitude of natural selection acting on the phenotypic traits we followed the standard model proposed by Lande & Arnold (1983). First, phenotypic traits were standardized to zero mean and unit variance while fitness metrics were relativized to the population mean. We calculated linear (s_i) and quadratic (c_i) selection differentials, that represent direct and indirect selection on a specific trait. In addition, selection gradients were estimated to determine the magnitude and sign of directional and stabilizing selection on a trait (β_i , γ_{ii}) or on correlated traits (γ_{ij}) independently of indirect effect by the other measured trait. Statistical significance of regressions was obtained through quasi-generalized linear models, with Poisson error distributions and log link function using R.

Significant phenotypic regression coefficients indicate that changes in floral traits affect

plant fitness. In addition, the magnitude and sign of selection differential and gradients show the strength and direction of phenotypic selection on floral traits. When these traits are central to a mutualistic interaction, such as flower length in pollination by hawkmoths, these analyses are key to understand the process of natural selection in coevolutionary partners. If we combine phenotypic analyses with correlations of interacting traits we can use them to reveal the pattern and possible causes mediating such selection. For traits that were significantly affected by phenotypic selection, we performed non-parametric regressions using cubic splines to depict multivariate associations with relative fitness metrics. To estimate the cubic splines we used the *mgcv* package (Wood, 2017) of R software. For each univariate spline, we fixed the covariate at its mean value. Smoothing parameters were obtained by minimizing the generalized cross-validation scores (Wood, 2008), and Bayesian standard errors were obtained according to Wood (2017). Finally, we visualized significant correlational selection acting on two traits plotting fitness surfaces using a thin-plate spline fit, a three-dimensional analog of cubic splines (Zuur *et al.*, 2009) using the *fields* package (Nychka *et al.*, 2017) of R.

2.4.4. Association of phenotypic selection with environment and proboscis length

We used generalized linear mixed-effects models with Poisson error distribution for each fitness metric and included a covariate term to determine whether the strength of phenotypic selection was influenced by environmental variables (i.e. latitude, elevation, precipitation and temperature), as well as by proboscis length (Bates *et al.*, 2015). The models included the interaction between the phenotypic traits (style length and filament length) as continuous terms and one covariate. Each interaction model was fitted separately. Population identity was included as a random term to account for differences in mean fitness across populations. Overdispersion was corrected nesting individuals within population. A significant interaction term indicated that selection on traits was associated with the covariance. We visualized significant interactions by plotting fitness surfaces and the covariance using the R package *visreg* (Breheny & Burchett, 2017).

3. Results

3.1. Association of floral traits with environmental variables

Mixed-effects models on each phenotypic trait showed that all phenotypic traits differed significantly among populations, among individuals and within individuals (Fig. 2). For all measured traits, the greater proportion of variance was explained by variation among individuals. Filament length, inflorescences number, and number of open flowers showed higher variability

among populations than within individuals.

There was a lack of association between the geographical and the phenotypic distance matrices with only 12% congruence (Protest: $m^2 = 0.88$, $P < 0.001$), thus indicating that phenotypic difference among populations could not be explained by geographic distance. Style and filament significantly increased in length with decreasing elevation (style: estimate=-0.28, $t=2.50$, $P=0.02$; filament: estimate=-0.57, $t=7.24$, $P<0.001$; Fig. 3a, b), and increasing temperature seasonality (style: estimate=0.57, $t=7.22$, $P<0.001$; filament: estimate=0.57, $t=7.22$, $P<0.001$), mean temperature of the wettest quarter (style: estimate=0.30, $t=2.99$, $P=0.007$; filament: estimate=0.54, $t=5.04$, $P<0.001$), and mean annual temperature (style: estimate=0.30, $t=3.02$, $P=0.007$; filament: estimate=0.49, $t=3.27$, $P=0.005$; Fig. 3a, b). Filament length was positively associated with increasing southern latitude (estimate: -0.19, $t=5.56$, $P<0.001$; Fig. 3b). Inflorescence number per plant was not significantly associated with environmental variables. During current samplings, mean style and filament lengths were significantly shorter, in mean 2.37 and 2.66 mm, respectively than previously recorded (style: estimate=2.84, $t=2.33$, $P=0.02$; filament; estimate=3.69, $t=3.07$, $P=0.002$; Fig. 4).

3.2. Proboscis length and its relationships with environmental variables

Proboscis length of previously recorded data showed positive relationships with latitude (estimate=-1.421, $t=-5.90$, $P<0.001$, Fig. 4a), temperature seasonality (estimate=0.004, $t=3.95$, $P<0.001$), mean temperature of the wettest quarter (estimate=0.08, $t=3.39$, $P<0.001$), mean annual temperature (estimate=1.29, $t=3.98$, $P<0.01$, Fig. 4b), and precipitation (estimate=0.11, $t=1.13$, $P=0.26$, Fig. 4c), but negative with elevation (estimate=-0.016, $t=-8.62$, $P<0.001$, Fig. 4d). During current samplings, mean proboscis length was significantly shorter, in mean 11.02 mm, than previously recorded ($t=6.43$, $P<0.001$; Fig. 4). This change in the guild mean is attributable to biases to shorter length both in the proboscis of each species and in species composition. All the species of the guild of pollinators showed lower proboscis length in the last sampling than in the historical sampling dates (Fig. 4e). In addition, the hawkmoth species with the longest mean proboscis length (*Maduca sexta*) was less frequently recorded (Fig. 4f), while, the species with the shortest mean proboscis length (*Lintneria maura*) was more frequently recorded during the last sampling period (Fig. 4f).

3.3. Floral traits association with proboscis length

The guild of hawkmoths showed higher proboscis length than style and filament lengths of *C. gilliesii* (Fig. 5a, b). There was a positive relationship of mean proboscis length with mean style

length (Pearson correlation test: $N=7$, $r=0.74$, $P=0.05$; Fig. 5c) and with mean filament length (Pearson correlation test: $N=7$, $r=0.96$, $P<0.001$; Fig. 5d) thus suggesting trait matching across populations.

3.4. Geographical variation of phenotypic selection

Opportunity for selection showed variation among populations with Rio Primero and Manantiales showing the highest variance values for seeds per fruits; Manantiales and Luro for fruits per inflorescence; Luro and Valle Hermoso for seeds*fruits per inflorescence, and Amaicha and Luro for seeds*fruits per plant metric of fitness (Table 1). For the population-wise selection models, Amaicha showed a significant disruptive selection differential on filament length for the seeds per fruit metric, but a stabilizing selection differential for number of fruits (Table 1). At Manantiales, filament length showed a significant positive directional selection differential through seeds*fruits fitness (Table 1). At Rio Primero, style length showed a negative directional selection differential for three of the four fitness metrics (Table 1). At the southernmost population (Luro), style length showed a disruptive selection differential for number of fruits, and a negative directional selection differential for the number of seeds*fruits per plant fitness metric (Table 1).

In the multivariate selection analysis, a significant negative directional selection gradient on style length but a positive one on filament length through seeds*fruits per inflorescence was detected at Manantiales (Fig. 6, Table 2). Also through this fitness metric, filament showed a stabilizing selection gradient for longer values (Fig. 6). In addition, a correlational negative selection gradient was observed between style and filament length for number of seeds per fruit, with individuals of highest fitness having long filaments and short styles at this population (Fig. 6, Table 2). At Rio Primero, a negative directional selection gradient on style length but a positive one on filament length through two fitness metrics was detected (Fig. 6, Table 2). At Luro, style length showed both negative directional selection and disruptive selection gradients for longer values through seeds*fruit per plant fitness metric (Fig. 6, Table 2). That is, there were two peaks of high fitness in the population; the highest of plants with the shortest styles and the lowest of plants with the longest styles (Fig. 6).

When populations were analyzed in a pooled model, negative selection on style length but positive on filament length were observed for both differentials and gradients, and were significant for some of the fitness metrics (Table S2 and S3).

3.5. Association of phenotypic selection with environment and proboscis length

Univariate and multivariate selection on filament length varied significantly with latitude,

elevation, temperature, and precipitation for seeds*fruits per inflorescence fitness (Table S2 and S3). For most of the fitness metrics, uni- and multivariate selection on style and filament length was associated with proboscis length (Table S2 and S3). Highest plant fitness was achieved in populations with matching interacting traits, i.e. long styles, long filaments, and long proboscis; or short styles, short filaments, and short proboscis (Fig. 7). When both environment and proboscis length contributed significantly on phenotypic selection on floral traits, proboscis length was more important than environment, excepting for fruits per inflorescence fitness metric (see R^2 of interaction models in Table S2 and S3).

4. Discussion

Trait variance partition analysis showed higher and significant variation among individuals than other levels, suggesting that the genetic component of variation prevails over the environmental component and that, consequently, traits are prone to heritable selection (Fox *et al.*, 2001). Pollinators often select for long floral traits, but if there is a trade off, such as a high metabolic costs of having larger flowers, then such size increases may be limited or even selected against given environmental conditions such as drought (e.g. Lambrecht & Dawson, 2007). For hawkmoths, it is known that proboscis length is correlated to body size which, in turn, is resource dependent, as larger larvae will moult to larger adults with longer probosces (Miller, 1997). Thus, mean proboscis length of pollinator guild appears to be significantly affected by environmental conditions as are the interacting floral traits. Therefore, the matching of interacting traits across populations may be conditioned by harsh environmental conditions that make long traits disadvantageous and setting a limit to the upper shift of optimal length by reciprocal directional selection (Maad, 2000). We observed a positive relationship between mean proboscis length of the local guilds and latitude, opposite to that observed when data is represented by species means (Miller, 1997). This opposite trend is explained by the low frequency of species bearing long probosces length across biogeographical regions (Johnson *et al.*, 2017). Consequently, the contribution of the long-proboscis individuals to community means may be masked by individuals bearing short probosces represented by many species at lower latitudes (Miller, 1997).

In the population-wise analyses, contemporary phenotypic selection on floral fertile parts was detected in about half of the studied populations of *C. gilliesii* and, when present, the target, mode and intensity of selection, varied spatially. The mode of selection was not consistent with the geographic pattern of flower trait variation as found before in other specialized plant-pollinator system (Ferreiro *et al.*, 2017). Consequently, current patterns of selection do not help to explain

the geographical variations of floral traits. However, there was significant negative directional selection on style length through three female fitness metrics in two populations. Selection gradients which measure selection on a trait by removing indirect effects of other traits showed that this is a direct effect through female fitness. The current pattern of selection against long flower parts is consistent with the detected reduction by about two mm in flower length during the past few years. Provided that variation in phenotypic traits is at least in part heritable, then trait reduction is expected to be a true evolutionary change (Brodie III *et al.*, 1995).

Analyses showed significant positive selection on filament length through two female fitness metrics in two populations where style length is negatively selected. It is not clear why a trait, expected to modulate male performance, should be affected by female fitness. Filament length is more likely under selection through male function (Campbell *et al.*, 2018), which should be further evaluated. Selection for short styles but long filaments was observed at two populations. Probably, the pollinator guild is selecting against herkogamy thus diminishing the difference between style and filaments resulting in a better match with interacting proboscis and maximizing the efficiency of pollen removal and deposition. However, selection against herkogamy is expected to meet the drawback due to an increase of autogamous and geitonogamous pollen deposition in this self-incompatible plant. In the context of the Geographical Mosaic Theory of Coevolution, these populations may be representing cold spots as, at least from the plant side, style length appears to be adjusting to new lower optima represented by the shorter probosces attained in the last years respect to previous records. Since coevolutionary cold spots are represented by communities where selection is one-sided or absent (Thompson, 2005), this hypothesis demands demonstrating that selection on the pollinator-side is not taking place in those populations. Obligate interactions have represented ideal study systems to estimate reciprocal adaptation and selection, as in flower parasitism by insects where fitness can be assessed through the number of eggs laid in the flowers (e.g. Thompson & Cunningham, 2002; Toju & Sota, 2006). Determining fitness in free-living pollinators in the wild and, particularly in nocturnal ones, is less easily achieved. To overcome this shortcoming in plant-pollinator systems, indirect fitness measures can be taken such as volume of nectar consumed (Pauw *et al.*, 2009). One possible approach for further studies could then be to estimate pollinator fitness through nectar consumption as conditioned by hawkmoth proboscis length relative to flower length.

Similar to Toju (2008) for the antagonistic interaction between the Japanese camellia and its obligate seed predator, we observed from the selection model on the pooled data-set that

proboscis length predominated as a modulator of phenotypic selection across populations over environmental variables. However, precipitation and temperature may have concordant or discordant modulating effects respect to proboscis length for some selection targets and fitness metrics. Mean precipitation was evidenced before as an important driver of selection on morphological traits of plants (Siepielski *et al.*, 2017). The pooled model of selection was consistent with the population-wise analyses, in that flowers with shorter styles are being significantly selected across the geographic range.

The detected reduction in the mean proboscis length of the hawkmoth guild of about 11 mm during the last few years is probably too large to be explained by selection on proboscis length of the species making up the guild. Instead, this change is rather attributable to changes in the relative abundance of species making up the guild. Consequently, selection is likely one-sided on plant traits and not reciprocal as expected in a hot spot.

Geographical variation of selection in free-living plant-pollinator systems have seldom been tested (but see Gómez *et al.*, 2009; Emel *et al.*, 2017; Ferreiro *et al.*, 2017). However, this knowledge is more advanced in the obligate yucca-yucca moth pollination mutualism (see Darwell *et al.*, 2017), in a number of plant-herbivore systems (e.g. Thompson & Cunningham, 2002; Gómez *et al.*, 2009), and in plant-seed predator interactions (e.g. Benkman, 1999; Toju & Sota, 2006; Toju & Sota, 2008; Siepielski & Benkman, 2010; Talluto & Benkman, 2014). These studies evidenced, as well as we did, that contemporary selection pattern results from conflicting selection pressures exerted by abiotic and biotic variables.

Our findings support previous few others about the geographical variations of selective pressures in a mosaic-like fashion of plant-pollinator interactions (Caruso *et al.*, 2003; Emel *et al.*, 2017; Ferreiro *et al.*, 2017). Differences in floral traits among populations were not spatially auto-correlated thus suggesting a patchy pattern of phenotypic variation. Therefore, variations in interacting trait length together with environmental variables are shaping the selective landscape at each population (Siepielski *et al.*, 2013). Considering that our results evidenced selection on filament length, in addition to style length, it should be evaluated if selection via male components of fitness, such as pollen removal, are particularly important in shaping interacting traits. Since we observed that univariate and multivariate phenotypic selection on style length was influenced by proboscis length and environmental variables, climate change may potentially shift selection patterns of the target plant species (Siepielski *et al.*, 2017). Our results suggest that mean proboscis length of the guild of hawkmoths is being shortened probably due to environmental

constraints thus changing local phenotypic selection on floral traits. The frequency of long- and short-proboscis species and mean proboscis length of the guild of hawkmoth change across populations and among years thus geographically exerting current variations of phenotypic selection on floral matching traits. Present results suggests that short term changes in environmental variables possibly caused by anthropogenic disturbance (see Alberti *et al.*, 2017) may be driving selection on floral traits at geographic and population levels because optima of reciprocal match are subtly shifted as a result in an ecological change.

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6. Author Contributions

F.S. and A.A.C. designed and performed the research, conducted fieldwork, analyzed the data, interpreted results, and wrote the manuscript; M.M. interpreted results and wrote the manuscript; A.M.R.P. and J.B.B. conducted fieldwork, interpreted results, and wrote the manuscript.

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Figure 1. Scenarios of coevolution of reciprocally matching traits in plant-pollinator interactions (the hawkmoth *Manduca sexta* and the brush-type flowered *Caesalinea gilliesii*, in the pictures)

along an axis of environmentally dependent phenotypic variation. The Gaussian curves represent the phenotype distributions for pollinator proboscis length (blue) and flower length (green). The dotted line represents the line of optimal length of trait matching along the environmental range. a) Hot spot area (red shading): both, flower and proboscis length, are subjected to positive directional evolutionary change (arrows) in response to reciprocal selection. Cold spot area (blue shading): b) Either interacting partners have reached their environmentally realizable optima thus configuring a scenario of stasis where variation is depleted and no selection takes place, c) or only one partner has reached the optimum (here plants) and the other partner is still adjusting through one-sided directional positive selection (arrow) to the optimum represented by the flower length to be matched. d) Environmental change may cause the environmental feasible optimum of one partner, here pollinator, to shift down (downward arrow) causing the other partner to readjust to a new optimum through one-sided negative directional selection (arrow).

Figure 2. Components of variance, expressed as percentage, between populations (n=7), among individuals (n=60) and within individuals (n=3) of each phenotypic floral trait of *Caesalpinia gilliesii* estimated with linear mixed-effects models. Significance levels estimated from REML are provided: *** P < 0.001, ** P < 0.01, * P < 0.05

Figure 3. Relationships of a) style and b) filament length of *Caesalpinia gilliesii* with environmental variables (latitude and elevation, mean annual temperature and mean annual precipitation) variables. Significance levels estimated from REML are provided: *** P < 0.001, ** P < 0.01, * P < 0.05, ns non significant.

Figure 4. Relationships of previously recorded data of proboscis length with (a) latitude, (b) elevation, (c) mean annual temperature, and (d) mean annual precipitation. Shaded bands represent 95% confidence intervals around solid line. Points represent observed mean values of proboscis length during the last sampling period, and previously recorded lengths. *** P < 0.001, ns non significant. (e) Proboscis length variation between two sampling periods for each hawkmoth species. Boxes show the 25% and 75% quartiles and the large dark line in the center of the box represents the median. Whiskers show minimum and maximum values within the boxplot range defined as $1.5 \times$ interquartile range. Values outside of the boxplot range are plotted as circles. (f) relative frequency, expressed as percentage, of each hawkmoth species for each sampling period.

Figure 5. a) Histograms of the distribution of proboscis length of the guild of hawkmoths (n=456),

and of style and filament lengths, respectively of *Caesalpinia gilliesii* (n=419); b) variation range of predicted proboscis, style and filament length across populations, error bars represent 95% confidence intervals; c) relationship between style and predicted proboscis length across populations; d) relationship between filament and predicted proboscis length across populations.

Figure 6. Significant phenotypic selection gradients on style length and filament length of seven populations of *Caesalpinia gilliesii*. Complete data are in Table 2. At all the populations, short styles (negative directional selection) but long filaments (positive directional selection) were selected. Mean filament lengths were selected for longer values at Manantiales (stabilizing selection). At Luro, extreme lengths of style were selected for longer values (disruptive selection). *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$. Shaded bands represent 95% confidence intervals around solid line.

Figure 7. Multivariate phenotypic selection on style and filament length in relation to proboscis length.

Supporting Information

Table S1. Population latitude, longitude, elevation and abiotic characteristics, as well as mean (\pm SD) of trait values of individuals of *Caesalpinia gilliesii* (n=60 per population).

Table S2. Interaction of environmental variables or proboscis length with univariate phenotypic selection on two flower traits in *Caesalpinia gilliesii* across seven populations.

Table S3. Interaction of environmental variables or proboscis length with multivariate phenotypic selection on two flower traits in *Caesalpinia gilliesii* across seven populations.

Table 1. Univariate phenotypic selection on two flower traits in *Caesalpinia gilliesii* across seven populations ordered by increasing latitude (n=60 in each population).

Population		Amaicha		Famatina		Valle Hermoso		Manantiales		La Calera		Rio Primero		Luro	
Fitness metrics	Trait i	Style length	Filament length	Style length	Filament length	Style length	Filament length	Style length	Filament length	Style length	Filament length	Style length	Filament length	Style length	Filament length
seeds/fruit	s_i (SE)	0.002 (0.066)	0.048 (0.073) .	0.074 (0.087)	0.167 (0.080)	0.280 (0.261)	0.226 (0.242)	-0.452 (0.300)	0.128 (0.288)	-0.088 (0.127)	0.019 (0.125)	-0.358 (0.094) *	0.022 (0.102)	-0.281 (0.276)	-0.028 (0.041)
	c_{ii} (SE)	-0.016 (0.119)	0.021 (0.119) *	-0.028 (0.106)	-0.016 (0.110)	-0.059 (0.395)	0.387 (0.360)	-0.160 (0.616)	-0.502 (0.469) .	-0.114 (0.219)	-0.069 (0.174)	-0.091 (0.142)	0.037 (0.180)	-0.171 (0.372)	-0.024 (0.058)
	Mean (I)	3.22 (1.04)		3.44 (1.52)		1.70 (2.02)		1.35 (2.07)		2.33 (1.89)		4.73 (2.53)		1.12 (1.87)	
fruits/inflorescence	s_i (SE)	-0.027 (0.074)	-0.028 (5.043)	0.070 (0.071)	0.126 (0.066)	0.124 (0.160)	0.108 (0.148)	-0.281 (0.154)	0.071 (0.149) .	-0.031 (0.052)	0.036 (0.051)	-0.255 (0.082)	-0.032 (0.086)	-0.039 (0.041)	-0.124 (0.277)
	c_{ii} (SE)	-0.005 (0.133)	-0.116 (0.132) *	0.067 (0.087)	0.027 (0.090)	-0.052 (0.240)	0.258 (0.219)	-0.047 (0.318)	-0.242 (0.243)	-0.052 (0.890)	-0.090 (0.071)	-0.061 (0.120)	0.054 (0.151)	0.003 (0.055) *	-0.194 (0.393)
	Mean (I)	0.12 (0.03)		0.10 (0.02)		0.26 (0.09)		0.69 (0.17)		0.16 (0.02)		0.25 (0.11)		0.56 (0.12)	
seeds*fruits/inflorescence	s_i (SE)	- (0.0003 (0.131)	0.062 (0.145)	0.265 (0.169)	0.324 (0.158)	0.058 (0.357)	-0.074 (0.330)	-0.750 (0.401)	0.355 (0.386) ***	-0.162 (0.170)	0.029 (0.167)	-0.476 (0.198)**	0.129 (0.201)	-0.407 (0.341)	-0.225 (0.342)
	c_{ii} (SE)	0.092 (0.236)	-0.122 (0.236)	0.215 (0.207)	-0.022 (0.217)	-0.106 (0.534)	0.648 (0.485)	-0.038 (0.831)	-0.530 (0.634)	-0.240 (0.293)	-0.254 (0.232)	-0.098 (0.281)	-0.009 (0.354)	-0.074 (0.461)	-0.2190 (0.488)
	Mean (I)	0.05 (0.002)		0.04 (0.002)		0.07 (0.04)		0.06 (0.03)		0.06 (0.03)		0.08 (0.01)		0.18 (0.27)	

seeds*fruits/plant	s_i	0.170	0.123	-0.089	0.099	0.291	0.608	-0.632	0.044	-0.092	0.204	-0.457	0.141	-0.689	-0.286
	(SE)	(0.259)	(0.287)	(0.204)	(0.194)	(0.797)	(0.703)	(0.430)	(0.413)	(0.238)	(0.231)	(0.183) *	(0.186)	(0.489)	(0.493)
														*	
	c_{ii}	-0.212	-0.360	-0.200	-0.067	-0.732	-0.480	-0.139	-0.630	-0.194	-0.310	0.067	0.200	0.057	-0.441
	(SE)	(0.466)	(0.465)	(0.247)	(0.257)	(1.175)	(1.069)	(0.882)	(0.672)	(0.408)	(0.322)	(0.260) .	(0.327)	(0.664)	(0.701)
	Mean	93.12 (21551)		46.56 (3189)		10.63 (1651)		14.57 (1760)		11.97 (541)		37.83 (2237)		19.14 (33)	
	(I)														

n= 60 individuals per population, “.” P<0.1, “*” P < 0.05, “**” P < 0.01, “***” P < 0.001

Standardized directional selection differentials (s_i), stabilizing/disruptive selection differentials (c_{ii}) and standard errors (SE) are given. Coefficients indicate the change in trait mean (s_{ii}) and variance (c_{ii}) in response to direct and indirect selection on the trait. Significant selection coefficients are in bold.

Table 2. Multivariate phenotypic selection on two flower traits in *Caesalpinia gilliesii* across seven populations ordered by increasing latitude (n=60 in each population).

Population		Amaicha		Famatina		Valle Hermoso		Manantiales		La Calera		Rio Primero		Luro		
Fitness metrics		Style length	Filament length	Style length	Filament length	Style length	Filament length	Style length	Filament length	Style length	Filament length	Style length	Filament length	Style length	Filament length	
Trait i	seeds/fruit	β_i (SE)	-0.023 (0.076)	0.060 (0.083)	-0.005 (0.095)	0.169 (0.090)	0.208 (0.318)	0.112 (0.294)	-0.543 (0.315)	0.284 (0.297)	-0.267 (0.204)	0.223 (0.199)	-0.403 (0.098) .	0.143 (0.095) .	- 0.4034 (0.404)	0.168 (0.402)
		γ_{ii} (SE)	-0.044 (0.154)	0.043 (0.191) .	0.019 (0.124) .	-0.218 (0.128)	- 0.3514 (0.530)	0.534 (0.700)	-0.067 (0.616)	-0.4880 (0.535)	0.058 (0.668)	0.269 (0.685)	0.149 (0.148)	0.152 (0.177)	0.225 (0.987)	-0.593 (0.925)
	fruits/inflorescence	β_i (SE)	-0.019 (0.085)	-0.018 (0.093)	0.014 (0.079)	0.119 (0.074)	0.085 (0.194)	0.064 (0.180)	-0.334 (0.161)	0.167 (0.152)	-0.155 (0.082)	0.154 (0.0801)	-0.271 (0.086)	0.050 (0.084)	-0.039 (0.060)	0.0001 (0.059)
		γ_{ii} (SE)	0.018 (0.172)	-0.168 (0.212) .	0.1260 (0.103)	-0.139 (0.106)	-0.261 (0.323)	0.345 (0.427)	-0.013 (0.316)	-0.277 (0.275)	0.280 (0.262)	0.229 (0.269)	0.094 (0.132)	0.119 (0.157)	0.039 (0.144)	-0.178 (0.135)
	seeds* fruits/ inflorescence	β_i (SE)	-0.034 (0.150)	0.080 (0.166)	0.141 (0.186)	-0.151 (0.402)	0.150 (0.434)	0.264 (0.178)	-0.952 (0.415) *	0.627 (0.392) ***	-0.480 (0.271)	0.395 (0.264)	-0.572 (0.206) ***	0.301 (0.200) *	-0.513 (0.500)	0.145 (0.497)
		γ_{ii} (SE)	0.142 (0.307)	-0.079 (0.379)	0.316 (0.238)	-0.495 (0.246) .	-0.730 (0.714)	0.794 (0.943)	0.139 (0.811)	-0.685 (0.706) *	0.512 (0.875)	0.587 (0.899)	0.299 (0.312)	0.230 (0.372)	0.469 (1.211)	-1.209 (1.135)
seeds* fruits/ plant	β_i (SE)	0.151 (0.296)	0.043 (0.328)	-0.169 (0.229)	0.171 (0.218)	-0.267 (1.052)	0.7612 (0.932) .	-0.711 (0.454)	0.248 (0.428)	-0.661 (0.373)	0.710 (0.364) .	-0.556 (0.189) **	0.308 (0.184) *	-1.018 (0.714) *	0.452 (0.711)	
	γ_{ii} (SE)	-0.146	-0.154	-0.109	-0.171	-1.180	-0.296	-0.174	-0.778	0.0284	-0.089	0.506	0.475	2.730	-1.614	

			(0.602)	(0.743)	(0.311)	(0.321)	(2.357)	(2.171)	(0.913)	(0.794)	(1.214)	(1.247)	(0.278)	(0.332)	(1.686)	(1.580)
Trait ij	seeds/fruit	Filament length γ_{ij} (SE)	-0.003 (0.142)		0.214 (0.111)		-0.041 (0.470)		- 0.7470 (0.423) **		-0.236 (0.632)		- 0.121(0.149)		0.041 (0.719)	
	fruits/inflorescence	Filament length γ_{ij} (SE)	0.041 (0.158)		0.180 (0.092)		0.002 (0.287)		-0.355 (0.217)		-0.314 (0.248)		-0.081 (0.133)		0.054 (0.105)	
	seeds* fruits/ inflorescence	Filament length γ_{ij} (SE)	-0.056 (0.283)		0.521 (0.212)		0.149 (0.634)		-0.965 (0.557) .		-0.727 (0.828)		-0.277 (0.314)		0.216 (0.883)	
	seeds* fruits/ plant	Filament length γ_{ij} (SE)	-0.167 (0.555)		0.108 (0.277)		0.296 (1.787)		-0.588 (0.626)		-0.192 (1.149)		-0.273 (0.280)		-0.812 (1.229)	

n= 60 individuals per population, “. ” P<0.1, “*” P < 0.05, “***” P < 0.01, “****” P < 0.001 Standardized directional selection gradients (β_i), stabilizing/disruptive selection gradients (γ_{ii}), correlational selection gradients (γ_{ij}), and standard errors (SE) are given. Coefficients indicate the change in trait mean (β_i) and variance (γ_{ii}) accounting for the indirect effects of correlated traits (γ_{ij}), in response to selection on the traits. Significant selection coefficients are in bold.













