Continuous variation in herkogamy enhances the reproductive response of *Lonicera implexa* to spatial variation in pollinator assemblages

Amparo Lázaro1*, Jaume Seguí1 and Luis Santamaría2

1 Global Change Research Group. Mediterranean Institute for Advanced Studies (IMEDEA; UIB-CSIC). C/ Miquel Marquès 21 07190 Esporles, Balearic Islands, Spain.

2 Doñana Biological Station (EBD-CSIC). C/ Américo Vespucio 26, Isla de la Cartuja 41092 Sevilla, Spain.

* Author for correspondence: amparo.lazaro@imedea.uib-csic.es
Abstract. Herkogamy, the spatial separation of sex organs in hermaphroditic plants, has been proposed as a mechanism to reduce self-pollination and the associated processes of inbreeding and gamete wastage. Longitudinal herkogamy is the most frequent type, with two subtypes: approach herkogamy (anthers below the stigma), which is associated with diverse pollinator arrays, and reverse herkogamy (anthers above the stigma), associated with specialized, long-tongued pollinators. By using a herkogamy index that varied continuously from negative (reverse herkogamy) to positive (approach herkogamy) values, we studied the effect of continuous variation in herkogamy on pollinator attraction, selfing capability, and plant fitness across three populations of Lonicera implexa differing in the relative abundance of long-tongued vs short-tongued pollinators. Reverse herkogamy was significantly more frequent in the population where long-tongued pollinators were dominant than in the other two populations. Agreeing with this, the main floral visitors of L. implexa individuals with small and large herkogamy index were, respectively, long-tongued and short-tongued pollinators. Spontaneous selfing was low and increased with increasing herkogamy index (i.e. with approach herkogamy), although most of it occurred when there was close distance between anthers and stigma. Fruit production was unrelated to the herkogamy index in the population with long-tongued pollinators, but it increased with approach herkogamy (higher herkogamy index) in the other two populations. In contrast, seeds of individuals with reverse herkogamy (smaller herkogamy indices) germinated better. In this species, continuous variation in herkogamy might function as a reproductive strategy, as different morphotypes might be favoured by different pollinator assemblages.

Keywords: corolla length; fruit set; Macroglossum stellatarum; pollinator visitation; seed germination; seed set; seed weight; selfing capability; stigma-anthers distance.
Introduction

Herkogamy is the spatial separation of sex organs in hermaphroditic plants and it is thought to have evolved in plants with different pollination modes to reduce self-pollination and to limit gamete wastage (Webb and Lloyd 1986; Barrett 2002a, 2002b, 2003). However, empirical evidence to support these hypotheses is still very scarce. In approach herkogamy, the stigma is presented above the anthers, whereas in reverse herkogamy, the anthers are located above a recessed stigma (Barrett 2003). In animal-pollinated plants, this means that pollinators contact the stigma first when visiting a flower with approach herkogamy, whereas they contact the anthers first when visiting one with reverse herkogamy (Barrett 2003). In animal pollinated plants, approach herkogamy is the most prevalent type of herkogamy and it is associated with a broad variety of pollinators. Reverse herkogamy is less common and it is supposed to be related to long-tongued pollinators, such as lepidoptera, when accompanied by long and narrow corolla tubes (Webb and Lloyd 1986; Barrett 2003), although not all plant species pollinated by lepidoptera display reverse herkogamy (Murcia 1990; Parra-Tabla and Bullock 2005).

While some plant species are polymorphic (i.e., they display two or several, discrete herkogamy morphs), most species are monomorphic, showing no discrete morphs and different extents of continuous variation in herkogamy. The majority of studies on herkogamy are focused on one type of polymorphism, the heterostyly, a reciprocal form of herkogamy in which there are two or threemorphs that differ reciprocally from one another in the same breeding population (Barrett et al. 2000; Barrett 2002b), as well as on other non-reciprocal polymorphisms related to heterostyly, but in different stages in their evolution (e.g. Pérez-Barrales and Arroyo 2010; Pérez-Barrales et al. 2018). The study of continuous variation in herkogamy in monomorphic species has received lesser attention, and has been mostly studied in relation to its effect on self-pollination (e.g. Parra-Tabla and Bullock 2005; Fishman and Willis 2007; de Vos et al. 2012) and to the developmental changes during anthesis that occur in some species (Barrett 2002a; Armbruster et al. 2009; de Vos et al. 2012).
2012). However, some other plant species exhibit continuous variation in the separation of sex organs from reverse to approach herkogamy that do not change with floral development (Forrest et al. 2011; Kulbaba and Worley 2008, 2012, 2013). These continuous variation deserves further attention, because the positions of sex organs can have profound implications for the mating biology of populations (Barrett 2002b), but also because a continuum including both approach and reverse herkogamy cannot be explained simply as a mechanism to reduce self-pollination (Kulbaba and Worley 2008). Instead, this variation could reflect contrasting selection by dissimilar pollinators. For instance, in Polemonium brandegei, a species pollinated by both hawkmoths and hummingbirds and that exhibit a continuous variation from reverse to approach herkogamy, it has been shown that hawkmoths select for reverse herkogamy whereas hummingbirds select for approach herkogamy (Kulbaba and Worley 2012, 2013). In this plant species, hawkmoth selection for reverse herkogamy seems to be mediated both by increased pollinator efficiency due to higher flower-pollinator match, and by increased pollinator attraction to this type of herkogamy, hypothesized to be mediated by lower obstruction of the opening of corolla tubes when fewer sex organs are exerted (Kulbaba and Worley 2012). On the other hand, selection may sometimes favour reduced herkogamy if a close proximity of stigma and anthers is necessary to ensure that small pollinators contact both sex organs (Sahli and Conner 2011), and to achieve spontaneous self-pollination when pollinators are scarce (Vos et al. 2012). However, very little is still known about the maintenance of these variations in the type and degree of herkogamy.

In this study, we document the continuous variation in herkogamy in Lonicera implexa, which goes from strong reverse herkogamy to strong approach herkogamy. Then, we assess whether this variation was simply a consequence of the variation in corolla length that the species exhibit (Lázaro et al. 2015; Lázaro and Santamaría, 2016), since in this species the stamen’s filament is short and fused to the inner part of a corolla tube but the stigma is free (Devesa and Ruiz, 2007). In addition, we evaluate the relationship between the degree and type of herkogamy and the visitation of long- vs. short-tongued pollinators, the capability of self-pollination, and plant fitness in three
populations differing in the importance of the diurnal hawkmoth, *Macroglossum stellatarum*, as pollinator. Our main hypothesis was that herkogamy diversity in this species may be maintained as a bet-hedging strategy that maximizes reproductive output across the range of variation in its pollinator environment, so that differences among sites in pollinator assemblages are related to differences in the degree and type of herkogamy. Specifically, we aimed to test whether: 1) the continuous variation in herkogamy in this species correlated with corolla dimensions; 2) long-tongued pollinators preferentially visit *L. implexa* flowers displaying reverse herkogamy; 3) spontaneous selfing increase as the distance between anthers and stigma was reduced; and 4) approach herkogamy is favored in populations with short-tongued pollinators whereas reverse herkogamy was favored when long-tongued pollinators (mainly hawkmoths) were abundant.

**Material and Methods**

**Study species**

The study species was the Mediterranean honeysuckle, *Lonicera implexa*, a climbing shrub that occurs in Southern Europe, mainly in the Mediterranean coast and south and central Spain. It flowers from late April to June, and its inflorescences bear approx. 6 hermaphroditic and zygomorphic flowers, which colour varies from pink (before opening) to white-yellowish (once open). Flowers have long corolla tubes that vary considerably in length, both within and among individual plants (ca. 14-47 mm, Devesa and Ruiz 2007; Lázaro et al. 2015; Lázaro and Santamaría 2016). In *L. implexa*, as in many other species with tubular flowers, the stamen’s filament is short (0-4.1 mm in Devesa and Ruiz 2007) and its base is fused to the inner part of a corolla tube, while the stigma is free and long, departing from a basal disk, and shows a broad range of variation (Devesa and Ruiz 2007). *L. implexa* suffers nectar robbing and it is pollinated by longue-tongued pollinators such as the hummingbird hawkmoth (*Macroglossum stellatarum*) and butterflies, but also by other
insects with shorter tongues, such as bees, flies and beetles; and the importance of the main pollinator differs among populations (Lázaro and Santamaría 2016). To our knowledge, there is no previous published information about the breeding system of this species. Each flower has 3-4 carpels that develop, when fecundated, into fleshy orange fruits (drupes) that are mature maturing by the end of August.

Study populations

We conducted our study in three populations of *L. implexa* separated by ca. 4 km from each other (range of distances: 3.3 to 4.5 km) and located in a mosaic of oak-pine forests and garrigue shrubland at the Tramuntana Mountains in Mallorca (Balearic Islands, Spain). The three populations were: (1) Establiments (39º 38' 54.56'' N, 2º 36' 25.84''), at ca. 160 m altitude; (2) Son Tries (39º 39' 38.19'' N, 2º 34' 32.66'' E), at ca. 260 m altitude; and (3) Banyalbufar (39º 40' 55.70'' N, 2º 31' 47.06'' E), at ca. 360 m altitude. While Establiments and Son Tries were located on southern slopes, Banyalbufar was on a northern slope. There was an increased importance of *Macroglossum stellatarum* as pollinator with increasing altitude in these populations (Lázaro and Santamaría 2016). While in Bayalbufar the great majority of flower visits were conducted by hawkmoths, in Son Tries both social bees and hawkmoths were equally important as pollinators and in Establiments hawkmoths were virtually absent and flower visitation was mainly conducted by small beetles (Lázaro and Santamaría 2016). Corolla tube length is a key trait in these populations since it is related to reward accessibility in the flowers and plant fitness (Lázaro *et al.* 2015; Lázaro and Santamaría 2016). In the two study populations where short-tongued insects are important pollinators (Son Tries and Establiments) decreased long corollas are selected for (see accessibility index, which
increased with the decrease of corolla length and the increase in corolla width; Lázaro and Santamaría 2016).

**Flower measurements**

In the spring 2012, we measured in the field the distance between the stigma and the anthers by using a digital calliper, and noted the relative position of the sex organs, i.e., whether the stigma was above (approach herkogamy) or below the anthers (reverse herkogamy). Measurements were conducted in 20 flowers belonging to 5 inflorescences (4 per inflorescence) in all individuals that flowered in the study populations (33, 38 and 38 in Banyalbufar, Establiments and Son Tries, respectively). These flowers were selected to be in the same developmental stage, i.e., completely open and receptive. In these same flowers we also measured the length and width of the corolla tube.

Using these data, we calculated a Herkogamy Index for each flower as a continuous variable that measures the distance between the stigma and the anthers and goes from negative values when the stigma is below the anthers (reverse herkogamy) to positive values, when the stigma is above the anthers (approach herkogamy).

**Pollinator visitation**

We conducted 312 observation periods of 15 minutes from 4th May to 4th June 2012 at the three populations, covering the complete flowering period of these three populations the study year. The observations were made separately on each focal, individually-marked plant (Banyalbufar: 27; Establiments: 36; Son Tries: 33), and conducted every day from 09.00 to 21:30 h, unless windy or cloudy/rainy conditions curtailed pollinator activity. Each day we observed all the three populations in different order, so all the populations were observed during all the day periods. We aimed at
observing every flowering individual at least during 3 observation periods, including the different periods of the day (average number of censuses conducted per individual: 3.74 ± 0.41, 3.03 ± 0.19, and 3.00 ± 0.16, for Banyalbufar, Establiments and Son Tries, respectively). The average number of flowers observed per individual plant in an observation period was 84.0 ± 30.9, 102 ± 18.1, and 86.1 ± 12.4, for Banyalbufar, Establiments and Son Tries, respectively. We complemented these diurnal censuses with 18 nocturnal censuses of 15 min at each population, conducted from 21:30–23:30, using a red light to reduce pollinator disturbance. None of these censuses found nocturnal moths pollinating the species; therefore, we assumed that nocturnal pollination might be of limited relevance in our study system (as reported for other Lonicera species, Gutián et al., 1993) and will report only on the results of the diurnal censuses.

During each observation period we noted the number of insect visits to the focal plant. A pollinator visit was defined to have occurred when there was contact between the visitor’s body and the reproductive organs (stigma or anther) of the flower. We categorized the flower visitors into long-tongued species (hawkmoths with 24-27 mm of proboscis length; Zlatkov et al., 2017, and butterflies - e.g. Cynthia cardui and Colias crocea, with proboscides of 14-15 mm; Corbet, 2000) and short-tongued species (social and solitary bees, small beetles, hoverflies and muscoid flies; << 10 mm proboscis length; authors’ unpublished data). After each observation period, we estimated the total number of open flowers of each individual plant, and used these data to estimate the total number of visits by both long and short-pollinators per plant. To evaluate differential preference by each of these groups of flower visitors, we calculated the proportion of long pollinators respect to the total pollinators visiting each individual plant during each observation census.
Capability of spontaneous self-pollination

In spring 2012, to estimate spontaneous selfing, we bagged two closed inflorescences in 18, 31 and 30 individuals in Banyalbufar, Establiments and Son Tries, respectively. In these same individuals, we marked other two inflorescences that were left open to natural pollination (controls). All the flowers of these inflorescences were counted (23.2 ± 2.1 and 24.9 ± 1.8 flowers per study individual in bagged and control inflorescences, respectively). At the beginning of July, when fruits started to initiate, we removed the bags to allow a correct development of the fruits. In September, we counted the fully developed fruits produced by these inflorescences. For each individual we obtained an Autogamy Index (AI) estimated as: AI = (FS_{selfing} – FS_{open-pollination}) / FS_{maximum}, where FS_{selfing} is the fruit set (fruits/flower) obtained from the bagging treatment, FS_{open-pollination} is the fruit set obtained from the open-pollination treatment in the same individual, and FS_{maximum} = FS_{selfing} when FS_{selfing} > FS_{open-pollination}, and FS_{maximum} = FS_{open-pollination} when FS_{open-pollination} > FS_{selfing}. The index ranges from -1 to 1, in an analogous manner as the ‘Relative performance of crosstypes (RP)’ index by Ågren and Schemske (1993). Positive values in our index indicate that spontaneous selfing outperform open-pollination, and negative values that open-pollination outperform selfing.

Although in our experiment the inflorescences open to natural pollination could also self-pollinate, lower fruit set in open-pollination as compared to selfed flowers could occur if inefficient pollinators decrease both outcrossing and selfing success. Selfing success could be reduced in the open-pollination treatment compared to the selfing treatment if there was: (1) efficient pollen removal from anthers, which deplete anthers’ pollen before self-pollination occurs; and/or (2) stigma clogging with pollen from other species (or incompatible genotypes), which blocks stigma receptivity before selfing takes place. As final fruit production was zero in many inflorescences (similarly in all the populations), to calculate the indices for each individual plant we merged the data of the two inflorescences of each treatment per individual.
**Plant fecundity**

In spring 2012, to estimate plant fecundity, we haphazardly selected and marked 5 inflorescences in each study individual (Banyalbufar: 33; Establiments: 36; Son Tries: 33) and counted the total number of flowers in those inflorescences. In September, when fruits were mature, we counted the number of fruits produced in these inflorescences and collected them for dissection in the laboratory, where the number of fully developed seeds in them was counted and each seed individually weighted (±1 mg). Weighted seeds were immediately sowed in watered germination trays at IMEDEA’s greenhouse, which plugs were labeled to identify individual seeds. Seed germination was followed every third day for over 2 months, until no new germinations occurred.

We used fruit set (fruits/flower), seeds/fruit, seed weight and seed germination as measures of plant fecundity.

**Statistical analyses**

All the analyses were conducted in R ver. 3.4.3 (R Core Team 2017). We used a General Linear Mixed Model (package lme4, function lmer; Bates et al. 2015) to assess how the herkogamy index varied among populations and with corolla tube length and width. The full model included population, corolla length and width and their interactions, because previous VIF analyses showed no collinearity between these predictor variables (all VIF values < 2; Zuur et al. 2009). The model included also individual and inflorescence nested within individual as random factors.

We conducted separate analyses to test the relationship between the herkogamy index and the attraction to long-tongued pollinators, the selfing capability (autogamy index, AI) and each fecundity variable (fruit set, seeds/fruit, seed weight and seed germination). We used Generalized Linear Models (package nlme; Pinheiro et al. 2018) for the models of selfing capability and fruit set, as individual plants were the sampling units; in these models we used the average herkogamy index
to characterize each individual. For the other response variables, we used Generalized Linear Mixed Models (package lme4; Bates et al. 2015) with individual plant as random factor in the models of proportion of visits by long-tongued pollinators and seeds/fruit; and plant, inflorescence and fruit as random factors in the models of seed weight and germination. Due to the nature of the data, we used: (1) binomial error distributions and logit link functions for the analyses of proportion of visits by long-tongued pollinators, fruit set and seed germination; (2) a gamma error distribution with log as a link function for the model of selfing capability; (3) a poisson error distribution and log link function for the analysis of seeds/fruit, after checking for the absence of overdispersion (Zuur et al. 2009); and (4) a gaussian error distribution and identity link function for the analysis of seed weight.

Full models included the herkogamy index as a continuous predictor variable, population as a fixed factor, and the interaction between population and the herkogamy index. Seed mass was added as an additional covariable in the model of seed germination. In all the cases, we used automatic model selection based on AIC (function dredge, package MuMIn; Barton 2018) to find the best models among the set of combinations of predictor variables and their interactions, setting the herkogamy index as a variable that should be always included in the models, since it was the main variable that we were interested to test. Significance of variables is based on Likelihood Ratio Tests (LRT). After each model, we ran Tukey posthoc tests to determine significant differences between levels of each significant categorical variable or interaction (function lsmeans, package lsmeans; Lenth 2016). Only best models are reported in the result section, where predicted means and model estimates are accompanied by their standard error.
Results

Variation in the herkogamy index and its relationship with corolla length and width

Herkogamy indices varied from -22.33 (reverse herkogamy) to 27.70 mm (approach herkogamy). Overall, the individuals from Banyalbufar showed significantly smaller herkogamy indices (i.e. higher reverse herkogamy) than those from Establiments and Son Tries (-3.16 ± 0.18, -0.40 ± 0.14 and 0.66 ± 0.14, for Banyalbufar, Establiments and Son Tries, respectively; $\chi^2 = 96.54$, df = 2, $P < 0.0001$). The relationship between corolla length and the herkogamy index differed also among populations (corolla length x population: $\chi^2 = 80.88$, df = 2, $P < 0.0001$). In Establiments and Son Tries the herkogamy index decreased significantly with corolla length (Fig. 1A, B), whereas in Banyalbufar, the herkogamy index was not related to corolla length (Fig. 1C).

Although there was considerable variation among- and within- individuals in each study site (Fig. 2), the populations differed clearly in the dominant morphotype: in Banyalbufar, most individuals showed reverse herkogamy; in Son Tries, most individuals showed approach herkogamy; while in Establiments, a fairly homogeneous proportion of individuals appeared across the whole range of values of the herkogamy index (Fig. 2).

Relationship between the herkogamy index and pollinator visitation

Reverse herkogamy was associated with more visits by long-tonged pollinators (i.e., decreasing the herkogamy index resulted in a considerable increase in the ratio of long-tongued vs. short-tonged pollinator visits) across the three populations (Table 1A; Fig. 3). Visits by long-tongued pollinators were more frequent in Banyalbufar than in the other two populations, although posthoc analyses showed that the differences were only significant between Banyalbufar and Establiments (0.48 ± 0.08, 0.22 ± 0.05 and 0.02 ± 0.01, for Banyalbufar, Son Tries and Establiments, respectively; Table
Visitation recorded by different pollinator groups is given in Table S1 in Electronic Supplementary Material.

Relationship between the herkogamy index and selfing capability

Spontaneous selfing events were few. They occurred only in 10 out of 79 bagged individuals and, in these 10 plants, the average number of selfed fruits was 1.8 ± 0.33. Selfing events took place at intermediate herkogamy values, i.e., when the separation between anthers and stigma was small (-4 to 4 mm; Fig. 4). The Autogamy Index (AI) could be calculated only for 45 individual plants, as the rest had no fruits either in the bagging or in control branches and were then excluded from the analysis (these individuals did not differ in herkogamy from those used to calculate AI (Range: -6.8 - 5.2 vs -6.6 -5.1, respectively; \( \chi^2 = 1.74, df = 1, P = 0.186 \)). Considering these 45 individuals, we only obtained 18 selfed fruits out of the 1044 flowers bagged (1.7%), while fruit set in the controls was 17% (190/1119). The Autogamy Index (AI) increased significantly with the herkogamy index (Table 1B), indicating that more spontaneous selfing occurred in individuals with increased approach herkogamy (Fig. 4).

Plant reproductive success

The herkogamy index had significant effects on both fruit set and seed germination. However, the effect of herkogamy on fruit set varied among populations (significant herkogamy index x population interaction; Table 1C). In Son Tries and Establiments, fruit set increased with the herkogamy index (i.e., higher approach herkogamy; Fig. 5A, B), while in Banyalbufar there was no relationship between the herkogamy index and fruit set (Fig. 5C). Populations also differed in overall fruit set, with Establiments having overall higher fruit set than the others (0.13 ± 0.029 vs. 0.08 ± 0.02 and 0.08 ± 0.02, for Establiments, Banyalbufar and Son Tries, respectively).
The number of seeds per fruit and seed weight were not significantly related to the herkogamy index (Table 1D, E). However, both variables differed significantly among populations (Table 1D, E). The fruits from Banyalbufar had significantly fewer seeds than those from Establiments and Son Tries (0.33 ± 0.08 vs. 1.43 ± 0.07 and 1.49 ± 0.1, respectively), that weighted lower (0.006 ± 0.0007 vs. 0.010 ± 0.0002 and 0.010 ± 0.0002 mg, for Banyalbufar, Establiments and Son Tries, respectively).

Seed germination (Table 1F; Fig. 6) decreased significantly as the herkogamy index increased, indicating that plants with flowers showing reverse herkogamy sired seeds that germinated better. Seed weight had an additional positive effect on germination (Table 1F).

Discussion

*Lonicera impplexa* showed a large variation in the degree and type of herkogamy. As hypothesized, reverse herkogamy was more frequent in the population where the flower-visitor community was dominated by long-tongued pollinators, – as compared to those with short-tongued pollinators. Likewise, long-tongued pollinators visited preferentially plants with reverse herkogamy, while short-tongued pollinators preferred plants displaying approach herkogamy across the three populations. Spontaneous selfing was low and, contrary to our expectations, increased with the herkogamy index, although most of it occurred when the distance between anthers and stigmas was very low. Fruit production was unrelated to herkogamy in the population where long-tongued pollinators dominated, while it increased with approach herkogamy in the other two populations. In contrast, individuals with reverse herkogamy sired seeds that germinated better. These results suggest that, in this system, continuous variation in herkogamy is maintained because the different morphotypes are favoured locally by the contrasting pollinator assemblages.
Continuous variation in herkogamy in *Lonicera implexa*

In monomorphic species, continuous variation in herkogamy values (distance between stigma and anthers) is not uncommon (e.g. Parra-Tabla and Bullock 2005; Fishman and Willis 2007; De Vos et al. 2012). Yet continuous variation spanning from reverse to approach herkogamy, as we found for *L. implexa*, has only been reported in a few species and with much smaller ranges of herkogamy values (Kulbaba and Worley 2008; Forrest et al. 2011).

In species as *L. implexa*, with individuals that differ considerably in corolla length (Lázaro et al. 2015; Lázaro and Santamaría 2016), variation in herkogamy may be dependent on variation in corolla length – e.g. if anthers change their position, relative to the stigma, due to changes in the corolla tube length. However, we have shown here that the relationship between herkogamy and corolla tube length varied among populations. Indeed, *L. implexa*’s herkogamy index decreased with corolla length in the two populations with short-tongued pollinators, while it was independent of corolla length in the population with long-tongued ones, suggesting that reverse herkogamy, which predominates among the latter population, is a trait directly selected by such long-tongued pollinators, independently of corolla length. The inter-related effects of long-tongued pollinators on both herkogamy and corolla length may be triggered by two complementary mechanisms: selection for reverse herkogamy due to positional effects (Kulbaba and Worley 2012) and selection for long corolla tubes due to resource partitioning effects (Santamaría and Rodríguez-Gironés 2015; see Fig.3 of Rodríguez-Gironés and Santamaría 2004 for a graphical summary of the process). Under such scenario, long corollas would attract long-tongued pollinators in populations where they are not dominant, triggering selection for reverse herkogamy in plants with long corollas (and thus an association between corolla length and herkogamy); while in assemblages dominated by long-tongued pollinators, they will visit all flowers – so that selection for reverse herkogamy would operate on all plants, independent of corolla length. Indeed, previous work on these populations support this hypothesis, as in Banyalbufar (the population where hawkmoths dominate), display
traits have a stronger relationship to *Macroglossum* visits than corolla tube length; while in Son Tries (the mixed population), corolla tube length and width are the determinant traits affecting the identity of pollinator visits (Lázaro and Santamaría 2016).

**Variation in herkogamy and its effects on self-pollination**

Herkogamy is hypothesized to evolve as a mechanism that reduces the negative effects of self-pollination: inbreeding depression in compatible species and gamete wastage in incompatible ones (Webb and Lloyd 1986; Barrett 2002a, 2002b, 2003). Indeed, most studies show that self-pollination increases as herkogamy (stigma-anther separation) decreases (Murcia 1990; Parra-Tabla and Bullock 2005; Fishman and Willis 2007). But decreasing self-pollination does not come free of risks: it results in failed reproduction whenever (or wherever) plant and/or pollinator abundances are low. In such cases, developmental reduction of herkogamy (i.e., a reduction in herkogamy through floral development) can make self-pollination unlikely at the beginning but increasingly likely at the end of floral development - thus enhancing reproductive assurance, via delayed autonomous selfing (Armbruster et al. 2002; de Vos et al. 2012). Although during sampling we did not detect any indication of developmental changes in herkogamy in this species, we restricted our measurements to a specific developmental stage, and therefore, we cannot evaluate this possibility with our data.

In this study, we found that the levels of spontaneous selfing were very low and always around small values of separation between stigma and anthers. Surprisingly, we also show that the capability of spontaneous self-pollination increased with approach herkogamy in *L. implexa*. This result contradicts Webb and Lloyd’s (1986) and Barrett (2003) assertions that for most floral architectures, approach herkogamy functions more effectively than reverse herkogamy in preventing intra-floral self-pollination and enhancing outcrossing rates. It is worth noting, however, that flower position could influence the probability of autogamy occurring in an approach
herkogamous flower. Although *L. implexa* flowers tend to be relatively erect or horizontal, rather than pendant, we did not collect data on flower position. Therefore, it is possible that changes in flower position affected the relationship between autogamy and approach herkogamy in this species. Alternatively, it could be that, in some cases, bagging provoked artefact contacts between stigmas and anthers (within or between flowers) or that the positive relationship between herkogamy index and self-pollination was a consequence of the differential effect of dichogamy in approach herkogamous flowers, not considered here (Cesaro et al. 2004). Besides, no selfing event was detected at stigma-anther distances larger than 4 mm; and, since these large values only occurred in the negative range of the herkogamy index for the individuals included in the experiment (Fig. 4), we cannot rule out that the correlation found is simply a consequence of an increase in selfing with decreasing stigma-anther distance (as reported by Murcia 1990; Parra-Tabla and Bullock 2005; Fishman and Willis 2007). Future studies might evaluate the role of intra-plant variation in the degree of herkogamy and selfing rates, as it might be that intra-plant variation in herkogamy helps to deal with the unpredictability of per-flower visitation rates by maintaining (low levels of) seed production in the absence of pollinator visits. In any case, our results suggest that *L. implexa*’s continuous variation in herkogamy (from approach to reverse herkogamy) cannot be explained simply as a mechanism to reduce self-pollination and increase outcrossing rates, similar to that reported by Medrano et al. (2012) for a species with approach herkogamy, *Narcissus longispathus*.

**Variation in herkogamy as a result of differences in pollinator environments**

Our study populations differed considerably in the abundance of *Macroglossum stellatarum* (Lázaro and Santamaria 2016). These differences among populations are not likely due to an inability of *Macroglossum* to fly across them, because this hawkmoth is a strong flier, able to cover very long distances during migration (Cánovas et al. 2015 and references therein), but rather by habitat
preferences and/or space use patterns. It is important to note, however, that this is an abrupt terrain where movement is relatively restricted; and that the three populations differ substantially in the orientation (thus probably in temperatures in early spring) and abruptness (from relatively flat terrain in Establiments to a strong slop in Banyalbufar). In addition, differences in pollinator distribution could reflect, at least partially, the observed differences in nectar accessibility (resulting from variations in corolla length and width; Lázaro and Santamaría 2016) – since L. implexa represents one of the most important nectar resources at these sites during that period of the year.

Among-population variation in herkogamy seemed well-fitted to the variation in pollinator assemblages. In the population where the hummingbird hawkmoth Macroglossum stellatarum was the main pollinator (Lázaro et al. 2015; Lázaro and Santamaría 2016), most L. implexa plants had flowers with reverse herkogamy (i.e. with lower herkogamy indices; Banyalbufar, Fig. 2). This is in agreement with the association between reverse herkogamy in flowers with long and narrow corolla tubes and pollination by longue-tongued lepidopterans (Webb and Lloyd 1986). Although the functional mechanisms behind this relationship are not totally clear (Barrett 2003), Kulbaba and Worley (2012) discussed increased potential for the narrow proboscis of hawkmoths to contact the sex organs within a narrow corolla tube. Alternatively, this association may be related to the fact that if stigmas are not deep enough within corolla tubes, they can be pollinated by short-tongued pollinators. In contrast, the populations where short-tongued pollinators were abundant (Lázaro et al. 2015; Lázaro and Santamaría 2016) individuals showed mostly approach herkogamy (Son Tries) or a broad range of herkogamy values (Establiments), consistent with our expectation that approach herkogamy is more common in populations with short-tongued pollinators (Webb and Lloyd 1986; Barrett 2003).

Previous studies on species with continuous variation from reverse to approach herkogamy have shown that the spatial and/or temporal variation in pollinator assemblage could be the mechanism behind the maintenance of such variability. In Polemonium brandegei, a species
pollinated both by hawkmoths and hummingbirds, hawkmoths selected for reverse herkogamy, whereas hummingbirds selected for approach herkogamy (Kulbaba and Worley 2012, 2013). In *Mertensia fusiformis*, seasonal changes in pollinator-mediated selection were suggested as the factor maintain variation in style length (Forrest et al. 2011). For differential selection to be the case in *L. implexa*, we needed either a higher preference of a particular pollinator for a type of herkogamy or a higher efficiency of this pollinator when visiting a type of herkogamy, with effects on plant fitness. In our study system, long-tongued pollinators (mostly hawkmoths) preferred to visit plants displaying reverse herkogamy (as in Kulbaba and Worley 2012). Kulbaba and Worley (2012) hypothesized that this preference might be mediated by an effect of exerted floral sex organs on hawkmoth foraging efficiency, since the opening of the corolla tube would be less obstructed; however, the exact mechanism still remains unknown. Differences among populations in visitation patterns, mediated both by pollinator preferences and differences among sites in pollinator assemblages, cascaded into plant reproductive success. In the population where long-tongued pollinators dominate, fruit production was unrelated to herkogamy, but plants with reverse herkogamy sired seeds that germinated better. This pattern suggests a scenario of reduced selfing and increased outcrossing in this population, resulting in moderate pollen transfer across the population (less seeds per fruit, on average) but higher seed quality in outcrossed fruits. In contrast, the two populations with short-tongued insects in the pollinator assemblage showed increased fruit production, but decreased seed quality (seed germination) in flowers with approach herkogamy. This is analogous to the variation in relative fitness of selfed (displaying low separation between anthers and stigma) and outcrossed (with high separation between anthers and stigma) lineages reported for *Datura stramonium* (Motten and Antonovics 1992; Stone and Motten 2002). In flowers with moderate levels of approach herkogamy, the contact of reproductive organs to exerted stigmas probably facilitates pollination by short-tongued visitors, but at the cost of increased selfing, both within plants and within flowers (Fig.4), and maybe also at the cost of biparental inbreeding, given the low distance that some short-tongued insects, such as beetles, might fly between flowers. The
overall higher quality of seeds (seeds that germinated better) from individuals with reverse herkogamy may be related to the fact that long-tongued pollinators are the most effective at transporting outcrossed pollen, as expected in this species with long and thin corolla tubes. This has been found in other species that display also a range from reverse to approach herkogamy (Kulbaba and Worley 2012). Indeed, a previous study conducted in the same populations (Lázaro and Santamaría 2016) supports also this idea, as it showed that where short-tongued insects are important pollinators (Son Tries and Establiments) decreased long corollas were selected for (see accessibility index), whereas this was not the case in the population where hawkmoths dominated (Banyalbufar).

It is however important to note that we only have studied one population dominated by hawkmoth pollinators. Without replicate populations we cannot completely rule out the possibility that the presence of the long-tongued pollinators and reverse herkogamy are coincidental. Also, we studied the effects on female fitness, thus not including potential effects of conflicting selection among gender functions, which tend to be weak but cannot be disregarded (Sahli and Conner 2011; Kulbaba and Worley 2012). In addition, it might be acknowledged that reproductive assurance may weaken, to some extent, pollinator-mediated selection on floral traits (Lázaro and Totland 2014), that the selective pressures on herkogamy may fluctuate between years as a consequence of changes in pollinator conditions, and that other interactions (e.g. protection from herbivory, nectar robbing and/or mechanical damage by other insects; Parra-Tabla and Bullock 2005; Lázaro and Santamaría 2016) can also modify the selective pressures. Therefore, our study only provides a first depiction of the processes and further studies are required to document the exact mechanisms behind the spatial variation in the relationship between herkogamy and pollinator assemblages. Moreover, the evolutionary importance of selection depends in part on whether variation of the attribute in question has a genetic basis. Although the heritability of continuous variation in style length has been shown in other species (Kulbaba and Worley 2008), studies on the heritability of this trait in *L. implexa* are needed.
Conclusions

In *L. implexa*, continuous variation from reverse to approach herkogamy might act as a reproductive strategy, optimizing seed quality when long-tongued pollinators are abundant, but increasing fruit set (through self-pollination and pollination by short-tongued insects) when they are rare. As a result, the continuous range of phenotypic variation in herkogamy is compatible with a geographical structured spatial pattern, in which different morphotypes seem to be favoured by the contrasting composition of the local pollinator assemblage.

Supporting information

Table S1. Frequency of visits to plants in total (Arrivals), by different flower-visitor groups (Long- and Short-tongued), and by pollinator orders (Orders) in 15 minutes observation periods, as well as plants’ number of open flowers (Flowers) and average Herkogamy index, at the three study *Lonicera implexa* populations.

Data

The data and the R-scripts utilized are available as Supporting Information.
Acknowledgements

The authors thank Clara Vignolo for her help during field work. AL was supported during field work by a ‘Juan de la Cierva’ contract financed by the Spanish Ministry of Science and Innovation. During the writing of this manuscript AL was supported by a Ramón y Cajal (RYC-2015-19034) contract from the Spanish Ministry of Science, Innovation and Universities, the Spanish State Research Agency, European Social Funds (ESF invests in your future) and the University of the Balearic Islands.

Contributions by the authors

AL and LS conceived the ideas; AL collected the data; AL and JS analysed the data; AL wrote the first draft, and JS and LS critically revised the manuscript.
Literatura cited

Ågren J and Schemske DW. 1993. Outcrossing rate and inbreeding depression in two annual monoecious herbs, Begonia hirsuta and B. semiovata. Evolution 47: 125–135.

Armbruster WS, Mulder CPH, Baldwin BG, Kalisz S, Wessa B, Nute H. 2002. Comparative analysis of late floral development and mating-system evolution in Tribe Collinsieae (Scrophulariaceae SL). America Journal of Botany 89: 37-49.

Armbruster WS, Hansen TF, Pélabon C, Pérez-Barrales R and Maad J. 2009. The adaptive accuracy of flowers: measurement and microevolutionary patterns. Annals of Botany 103: 1529–1545.

Barrett SCH, Jesson LK and Baker AM. 2000. The evolution and function of stylar polymorphisms in flowering plants. Annals of Botany 85: 253-265.

Barrett SCH. 2002a. Sexual interference of the floral kind. Heredity 88: 154-159.

Barrett SCH. 2002b. The evolution of plant sexual diversity. Nature 3: 274-284.

Barrett SCH. 2003. Mating strategies in flowering plants: the outcrossing-selfing paradigm and beyond. Proceedings of the Royal Society of London series B 358: 991-1004.

Barton K. 2018. MuMIn: Multi-Model Inference. R package version 1.40.4. https://CRAN.R-project.org/package=MuMIn

Bates D, Maechler M, Bolker B and Walker S. 2015. Fitting Linear Mixed-Effects Models Using lme4. Journal of Statistical Software 67(1): 1-48. doi:10.18637/jss.v067.i01.

Cánovas JL, Jiménez JF, Mota JF and Sánchez-Gómez P. 2015. Genetic diversity of Viola cazorlensis Gand., an endemic species of Mediterranean dolomitic habitats: implications for conservation. Systematics and Biodiversity 13(6): 571-580.
Cesaro AC, Barret SCH, Maurice S., Vaissiere BE, and Thompson JD. 2004. An experimental evaluation of self-interference in *Narcissus assoanus*: functional and evolutionary implications. *Journal of Evolutionary Biology* 17: 1367–1376.

Corbet SA. 2000. Butterfly nectaring flowers: butterfly morphology and flower form. *Entomologia Experimentalis et Applicata* 96: 289–298.

De Vos JM, Keller B, Isham ST, Kelso S and Conti E. 2012. Reproductive implications of herkogamy in homostylyous primroses: variation during anthesis and reproductive assurance in alpine environments. *Functional Ecology* 26: 854–865.

Devesa JA and Ruíz T. 2007. *Lonicera*. CLV Caprifoliaceae-Carifoliaceae. In: *Flora Iberica*, vol. XV Rubiaceae-Dipsacaceae. Devesa, J. A., Gonzalo, R. and Herrero, A. (eds). Dpto. de Publicaciones del CSIC, pp: 167-190.

Fishman L and Willis JH. 2007. Pollen limitation and natural selection on floral characters in the yellow monkeyflower, *Mimulus guttatus*. *New Phytologist* 177: 802-810.

Forrest JRK, Ogilvie JE, Gorischeck AM and Thomson JD. 2011. Seasonal change in a pollinator community and the maintenance of style length variation in *Mertensia fusiformis* (Boraginaceae). *Annals of Botany* 108: 1-11.

Guitián PJ, Guitián J and Navarro L. 1993. Pollen transfer and diurnal versus nocturnal pollination in *Lonicera etrusca*. *Acta Oecologica* 14: 219 – 227.

Kulbaba MW and Worley AC. 2008. Floral design in *Polemonium brandegeei* (Polemoniaceae): genetic and phenotypic variation under hawkmoth and hummingbird pollination. *International Journal of Plant Sciences* 169: 509-522.
Kulbaba MW and Worley AC. 2012. Selection of floral design in *Polemonium brandegeei* (Polemoniaceae): female and male fitness under hawkmoth pollination. *Evolution* **66**: 1344-1359.

Kulbaba MW and Worley AC. 2013. Selection on *Polemonium brandegeei* (Polemoniaceae) under hummingbird pollination: in opposition, parallel, r independent of selection by hawkmoths? *Evolution* **67**: 8: 2194–2206.

Lázaro A, Vignolo C and Santamaría L. 2015. Long corollas as nectar barriers in *Lonicera implexa*: interactions between corolla tube length and nectar volume. *Evolutionary Ecology* **29**: 419–435.

Lázaro A and Santamaría L. 2016. Flower-visitor selection on floral integration in three contrasting populations of *Lonicera implexa*. *American Journal of Botany* **103** (2): 1–12.

Lázaro A and Totland Ø. 2014. The influence of floral symmetry, dependence on pollinators and pollination generalization on flower size variation. *Annals of Botany* **114**: 157–165.

Lenth RV. 2016. Least-squares means: The R package lsmeans. *Journal of Statistical Software* **69**: 1–33. https://doi.org/10.18637/jss.v069.i01

Medrano M, Requerey R, Karron JD and Herrera CM. 2012. Herkogamy and mate diversity in the wild daffodil *Narcissus longispathus*: beyond the selfing–outcrossing paradigm in the evolution of mixed mating. *Plant Biology* **14**(5): 801-810.

Motten AF and Antonovics J. 2002. Determinants of outcrossing rate in a predominantly self-fertilizing weed, *Datura stramonion* (Solanaceae). *American Journal of Botany* **79** (4): 419-42.

Murcia C. 1990. Effect of Floral Morphology and Temperature on Pollen Receipt and Removal in *Ipomoea trichocarpa*. *Ecology* **71**(3): 1098-1109.
Parra-Tabla V and Bullock S. 2005. Ecological and selective effects of stigma-anther separation in the self-incompatible tropical tree *Ipomea wolcottiana* (Convolvulaceae). *Plant Systematics and Evolution* 252: 85-95

Pérez-Barrales R and Arroyo J. 2010. Pollinator shifts and the loss of style polymorphism in *Narcissus papyraceus* (Amaryllidaceae). *Journal of Evolutionary Biology* 23: 1117–1128.

Pérez-Barrales R, Abarca CA, Santos-Gally R, Schiestl FP, Arroyo J and Thompson JD. 2018. The function of the floral corona in the pollination of a Mediterranean style dimorphic daffodil. *Plant biology* 20: 118-127.

Pinheiro J, Bates D, DebRoy S, Sarkar D and R Core Team. 2018. nlme: Linear and Nonlinear Mixed Effects Models. *R package version 3.1-137*, <URL: https://CRAN.R-project.org/package=nlme>.

R Core Team. 2017. *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/.

Sahli HF and Conner JK. 2011. Testing for conflicting and non-additive selection: floral adaptation to multiple pollinators through male and female fitness. *Evolution* 65: 1457-1473.

Santamaría L and Rodríguez-Gironés MA. 2015. Are flowers red in teeth and claw? Exploitation barriers and the antagonist nature of mutualisms. *Evolutionary Ecology* 29 (3): 311-322.

Stone JL and Motten AF. 2002. Anther-stigma separation is associated with inbreeding depression in *Datura stramonion*, a predominantly self-fertilizing annual. *Evolution* 56 (11): 2187-2195.

Rodríguez-Gironés MA and Santamaría L. 2004. Why are so many bird flowers red? *PLoS biology* 2 (10): e350.

Webb CJ and Lloyd DG. 1986. The avoidance of interference between the presentation of pollen and stigmas in angiosperms. II. Herkogamy. *New Zealand Journal of Botany* 24: 163-178.
Zlatkov B, Beshkov S and Ganeva T. 2017. *Oenothera speciosa* versus *Macroglossum stellatarum*: killing beauty. *Arthropod-Plant Interactions*. https://doi.org/10.1007/s11829-017-9588-3.

Zuur AF, Ieno EN, Walker NJ, Saveliev AA and Smith GM. 2009. *Mixed effects models and extensions in ecology with R*. Springer, New York.
Figure legends

Figure 1 Relationship between the herkogamy index and corolla length in each study population. The average (± SE) partial residuals of the model for each individual plant are shown. Lines represent the estimates for the best model in each population. Interaction herkogamy index * population: $P < 0.0001$.

Figure 2 Boxplots showing the herkogamy index for each study individual. Red: individuals from Banyalbufar; Blue: individuals from Establiments; Green: individuals from Son Tries.

Figure 3 Relationship between the herkogamy index and the proportion of long-tongued visitors per observation census. Lines represent the estimates for the best model and the dots the average (± SE) of the proportion of long-tongued visitors per observation census on an individual plant. Herkogamy index: $P = 0.007$.

Figure 4 Relationship between the herkogamy index and the autogamy index ($AI = (FS_{self} - FS_{open-pollination}) / FS_{maximum}$, where FS is fruit set). Line represents the estimate for the best model and the dots represent the estimates of the autogamy index for each study plant. Herkogamy index: $P = 0.024$.

Figure 5 Relationships between the herkogamy index and fruit set in in each study population. Lines represent the estimates for the best model in each population and the dots the values of fruit set for each individual study plant. Interaction herkogamy index * population: $P < 0.0001$.

Figure 6 Relationships between the herkogamy index and seed germination. Line represents the estimate for the best model and the dots the proportion of germinated seed for each individual plant. Herkogamy index: $P = 0.012$. 
Table 1 Results of the best models showing the relationship between the herkogamy index and insect visitation, capability of spontaneous selfing, and fecundity variables. For each variable that appear in the best models, the $\chi^2$, the degrees of freedom (df) and the $P$-value are shown. The variables involved in significant interactions were also included in the full model. Significant values are marked in bold. The herkogamy index was fixed while selecting the best models, because we were specifically interested in testing its effect on the response variables.

| Model | Variable | $\chi^2$ | df | $P$  |
|-------|----------|----------|----|------|
| A)    | Proportion of visits of long-tongue pollinators | Herkogamy index | 7.33 | 1 | 0.007 |
| B)    | Autogamy Index | Herkogamy index | 5.07 | 1 | 0.024 |
| C)    | Fruit set | Herkogamy index * Population | 32.20 | 2 | < 0.0001 |
|      |          | Population | 56.42 | 2 | < 0.0001 |
| D)    | Seeds per fruit | Herkogamy index | 1.10 | 1 | 0.294 |
|      |          | Population | 16.59 | 1 | 0.0003 |
| E)    | Seed weight | Herkogamy index | 1.08 | 1 | 0.299 |
|      |          | Population | 9.35 | 1 | 0.009 |
| F)    | Seed germination | Herkogamy index | 6.36 | 1 | 0.012 |
|      |          | Seed weight | 27.57 | 1 | < 0.0001 |
Figure 6