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Long-legged bees make adaptive leaps: linking adaptation to coevolution in a plant–pollinator network

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Adaptation is evolution in response to natural selection. Hence, an adaptation is expected to originate simultaneously with the acquisition of a particular selective environment. Here we test whether long legs evolve in oil-collecting *Rediviva* bees when they come under selection by long-spurred, oil-secreting flowers. To quantify the selective environment, we drew a large network of the interactions between *Rediviva* species and oil-secreting plant species. The selective environment of each bee species was summarized as the average spur length of the interacting plant species weighted by interaction frequency. Using phylogenetically independent contrasts, we calculated divergence in selective environment and evolutionary divergence in leg length between sister species (and sister clades) of *Rediviva*. We found that change in the selective environment explained 80% of evolutionary change in leg length, with change in body size contributing an additional 6% of uniquely explained variance. The result is one of four proposed steps in testing for plant–pollinator coevolution.

1. Introduction

Long-legged *Rediviva* bees are among the most unusual insects. They live in the temperate parts of Southern Africa, where they use their elongated front legs to extract oil from long-spurred flowers [1,2] (figure 1). An unusual trait, such as long legs, invites the hypothesis of adaptation—the idea that the trait was produced by a particular selective environment, in this case long-spurred flowers. The adaptation hypothesis makes two key predictions: (i) that the trait is more advantageous than its antecedent in a particular selective environment, and (ii) that the trait evolved in that selective environment [3]. In other words, a trait is an adaptation if it serves a purpose and originated for that purpose.

The first prediction concerns utility: is it an advantage to have longer legs? There can be little doubt that *Rediviva* use their long legs to great advantage (figure 1c), but a more formal test of utility requires measuring performance relative to shorter-legged individuals (or species) in the same selective environment. Selection on leg length has not been studied in bees, but selection has been studied in another pollinator, a long-proboscid fly, which also interacts with long-tubed flowers [4]. This study found that individuals with longer appendages obtained more reward from long-tubed flowers, and the same mechanism is likely to favour longer legs in *Rediviva*.

The second prediction, which is the focus of this paper, concerns genesis. Did the trait originate simultaneously with the acquisition of a particular
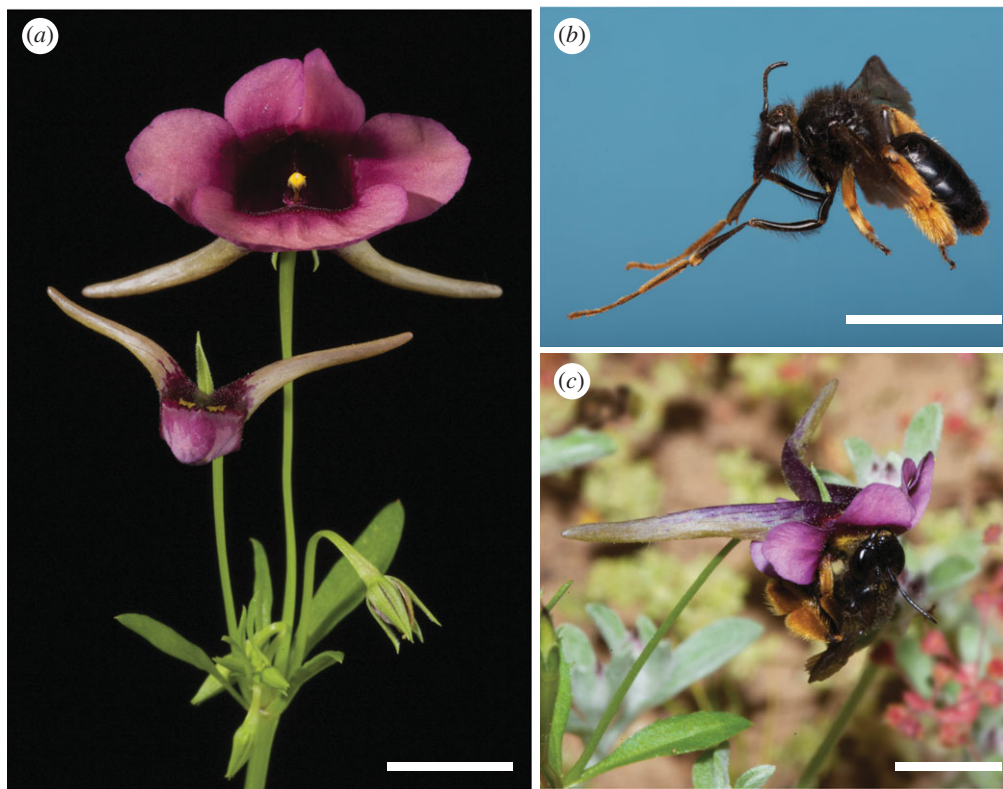
Figure 1. (*a*) *Diascia whiteheadii* with an open flower and several buds. The twin spurs contain oil. (*b*) *Rediviva longimanus* females have disproportionately long legs with dense hairs on the tarsi. (*c*) *R. longimanus* uses its long front legs to collect oil from the equally long spurs of *D. whiteheadii*. Cederberg, Greater Cape Floristic Region, South Africa. Scale bars, 1 cm. Photos by Anton Pauw.

selective environment? Using a phylogenetic tree for a group of organisms, change in traits and environments can be inferred by contrasting the descendants of a common ancestor [5]. The method simply requires calculating the difference in trait value and selective environment value between sister species pairs. The same procedure can be followed for sister clades, each of which is represented by the average value of the species it contains. A phylogeny of N species will produce $N - 1$ contrasts, and these can be used to test whether the amount of divergence in traits is correlated with the amount of divergence in selective environments. A positive association would be consistent with the idea that the trait originated as a result of the hypothesized selective environment [6].

Here we use this method to test for a significant association between evolutionary change in leg length in *Rediviva* and change in the hypothesized selective environment imposed by long-spurred flowers. To quantify the selective environment, we drew a network of the interactions between *Rediviva* species and oil-secreting plant species. We weighted the interactions by frequency, i.e. the number of bees captured per host plant species. The selective environment for each bee species was the weighted average spur length of its host plant species. To quantify evolutionary change in leg length and selective environment, we calculated the difference in leg length and the difference in average floral spur length for sister species and clades of *Rediviva*, and correlated these contrasts. We expected a positive linear relationship.

Because the traits of a species are correlated, adaptation in one trait causes necessary changes in other traits, which may falsely be identified as adaptations [7]. Thus, when testing for adaptation, analyses should be conducted using multiple traits simultaneously. Here we include body size with leg length in correlations with the selective environment.

2. Material and methods

(a) Study species

Rediviva (Melittidae) contains 26 species of solitary bees [8–10]. In all species, the females have a dense piles of plumose hairs on their front tarsi with which they collect floral oil. The oil is mixed with pollen to form a ball of food for the larvae [11], and the walls of the underground nest cells are also slicked with oil. A well supported phylogenetic hypothesis was available for 19 of the species [12] (electronic supplementary material, figure S1). This phylogeny, and earlier work [13], shows that *Redivivoides*, with seven species, is embedded within *Rediviva*, but lacks oil-collecting hairs [14]. We excluded them because their selective environment (non-oil-secreting flowers) is qualitatively different from that of *Rediviva*.

Rediviva bees collect oil from plants in the Scrophulariaceae, Orchidaceae, Stilbaceae and Iridaceae [15–18]. The alpha taxonomy of the largest genus, *Diascia* (ca 70 spp., Scrophulariaceae), is unresolved but temporary names for new species have been published along with brief descriptions, and associated specimens are housed in the Compton Herbarium [19,20]. We followed this taxonomy, along with additional sources [21].

(b) Traits

Measurements of leg length and body length were taken from the literature [8,9,16,22–24]. Additional measurements were made for *Rediviva steineri* using established methods [22]. Sample sizes ranged from eight to 59 individuals, except for the poorly known *R. steineri* ($n = 2$). Leg length was the mean leg length per species. For the highly variable *Rediviva neliana*, measurements were taken from the population with the longest legs (Mountain Zebra National Park). To visualize divergence in leg length between sister species we arranged the *Rediviva* species along an axis of increasing leg length and linked them with

lines representing phylogeny (R function 'phenogram'; package 'phytools'; R statistical environment) [25,26].

(c) Selective environment

To quantify the environment within which *Rediviva* front legs evolved, we documented the network of interactions between oil-secreting plants and female *Rediviva*. The data were derived mainly from the collection of the South African Museum, in particular those made by Vin Whitehead and Kim Steiner (3758 *Rediviva* specimens, mainly captured on *Diascia*). These were supplemented with data from the literature (31 specimens) [23,27], and our collections (240 specimens, including many captured on orchids). Collectors followed a plant-centred, representative sampling scheme [28] (V Whitehead 2002, personal communication): oil-secreting plant species were targeted for observation and *Rediviva* were captured on the focal plant species with insect nets until diversity levelled off. All plant species were observed at a minimum of three sites, in a minimum of 3 years.

The data were entered into an interaction matrix with *Rediviva* species as rows and plant species as columns. The cells were populated with interaction frequency, i.e. the number of individual bees captured on a particular plant species. To visualize the web of interactions, the data were plotted as a bipartite network in which *Rediviva* species were linked to plant species by lines of varying thickness, which represent interaction frequency (R function 'plotweb'; package 'bipartite') [29].

Spur length data were obtained from the literature [19–23,30–34]. For species with twin spurs (or sacs) into which the bee inserts only its front legs, spur length was the depth of these structures. For pouched species that accommodate the entire body of the bee, and for species with exposed elaiophores, spur length was zero. The selective environment for each bee species was the average spur length of the interacting plant species weighted by interaction frequency. To visualize the selective environment and its relation to bee traits, we plotted the location of all 4029 interactions in relation to axes of spur length and leg length (R function 'sunflowerplot').

(d) Quantifying evolutionary change in traits and environment

To quantify evolutionary change in leg length, selective environment and body size, we calculated phylogenetically independent contrasts (R function 'pic'; package 'ape') [35]. Contrasts for sister species were calculated simply by subtraction, and may be negative or positive. To calculate contrasts between internal nodes, average trait values were first calculated at each node, proceeding down from the tips. Contrasts were then calculated as the differences between the average trait values of sister clades. Note that trait values at internal nodes are clade averages, not reconstructed ancestral traits [36]. Contrasts infer evolutionary change by moving across the terminals of the tree. Alternative methods of testing adaptation infer change by moving from ancestor to descendant (e.g. [37]). We use contrasts in preference to these methods because, if adaptation is rapid relative to the rate of speciation, it will not be possible to reliably estimate ancestral traits or environments [38, p. 84]. The contrasts method has the added advantage that it is robust to phylogenetic uncertainty [39].

If traits diverge gradually over time, contrasts involving longer periods of time will be larger and will have greater weight in statistical analyses. Thus, contrasts generally need to be transformed to take into account differences in divergence time [40]. To test whether transformation is necessary, we regressed the absolute values of the contrasts against the square root of the sum of their branch lengths. A positive correlation would indicate the need for transformation.
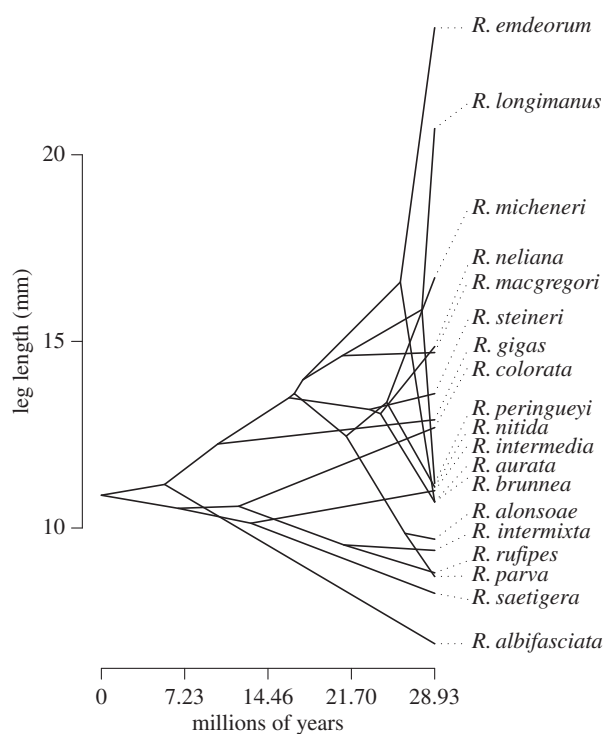

Figure 2. A diagram of leg length evolution in *Rediviva* shows that the bees with the longest legs are each closely related to short-legged sister species. Lines represent relatedness and the position of species along the *y*-axis represent foreleg length (mm). Ancestral states are maximum-likelihood estimations with considerable uncertainty and are not used in any tests.

(e) Correlation analysis

To test whether evolutionary change in leg length is associated with change in selective environment and change in body size, we used multiple linear regression with contrast in spur length and body size as simultaneous predictors of evolutionary change in leg length (R function 'lm'). The intercept was set to zero because contrasts are expected to be centred around zero due to the arbitrary direction of subtraction during their calculation [40]. After the analysis we tested whether the residuals were normally distributed (R function: 'shapiro.test'). Additionally, we calculated the partitioning of variance between the two predictor variables using their contribution to the total sum of squares, and plotted the model in three dimensions (R function 'plotPlane'; package 'rockchalk'). Then, we repeated the analysis using a reduced dataset form which contrasts at internal nodes were excluded (i.e. only contrasts between the seven sister species pairs were included). These contrasts had stronger phylogenetic support and were not affected by the exclusion of *Redivivoides* (electronic supplementary material, figure S1).

3. Results

(a) Traits

Average leg length in *Rediviva* varied from 6.9 to 23.4 mm, whereas body size ranged from 9 to 16.8 mm (electronic supplementary material, table S1). When we arranged the species along an axis of leg length and connected them using the branches of the phylogeny, the tree became tangled, as multiple lineages crossed over into the same area of morphospace (figure 2). Sister taxa often differed radically in leg length. In particular, the three species with the longest legs were each most closely related to an allopatric, short-legged
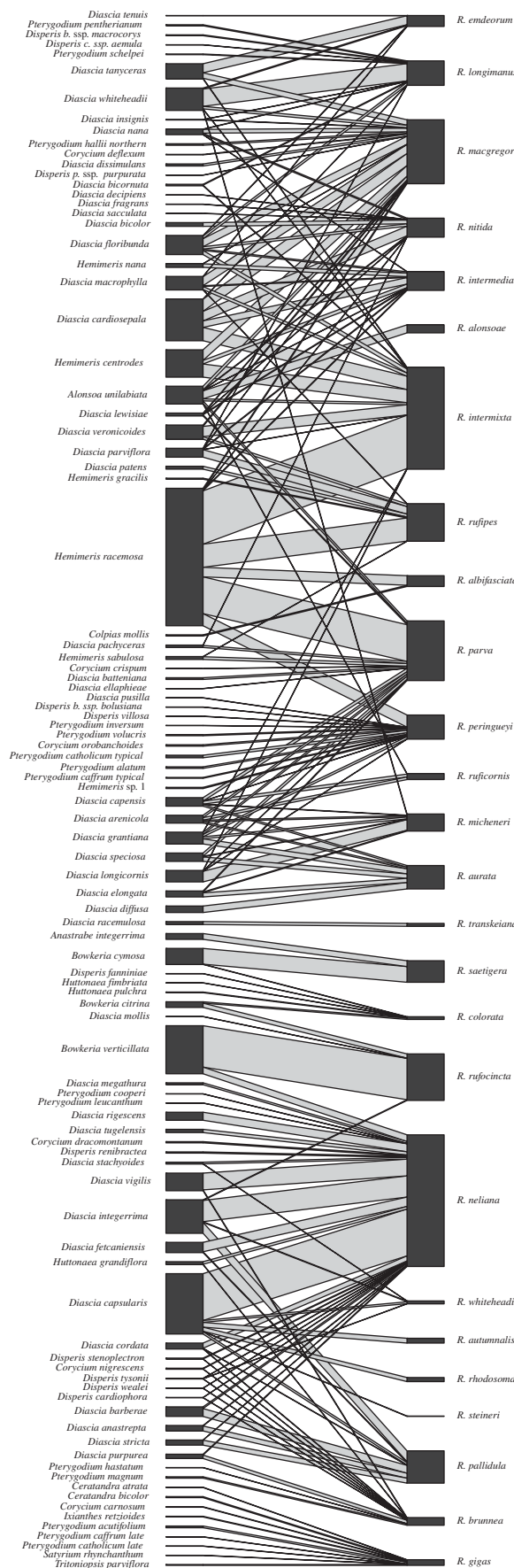
Figure 3. The selective environment in which *Rediviva* leg length evolves is represented as a bipartite network of 4029 interactions among female bees and oil-secreting host plants. Line thickness represents the number of bee specimens captured per plant species. Bees were collected throughout South Africa.
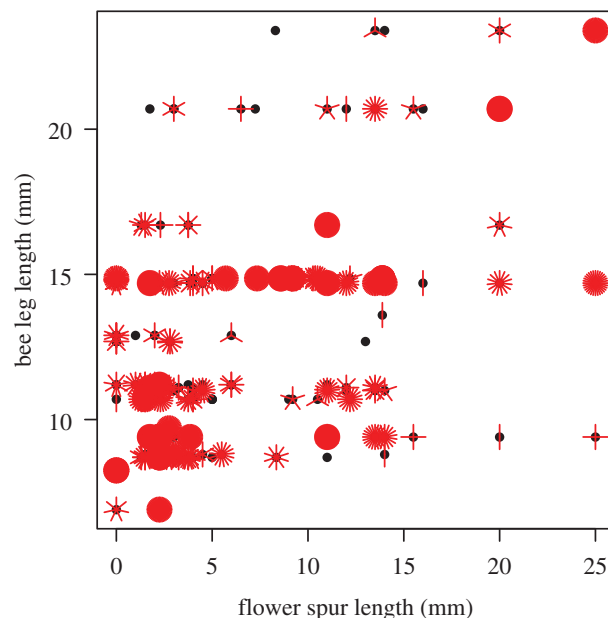

Figure 4. The trait – environment relationship between *Rediviva* foreleg length and the spur length of the host plants shows frequent mismatching. Points represent 4029 interactions. Multiple points are plotted as 'sunflowers' with multiple 'petals'. (Online version in colour.)
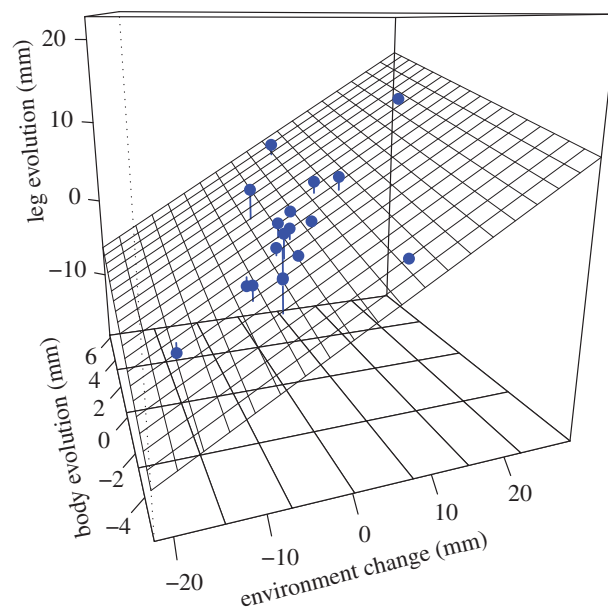

Figure 5. Evolutionary change in leg length in *Rediviva* is associated with change in the selective environment imposed by long-spurred flowers. Evolutionary change in body size explains additional variation in leg length evolution. The points represent 18 phylogenetically independent contrasts between *Rediviva* sister species, or sister clades (electronic supplementary material, figure S4). The linear model prediction (table 1) is represented as a plane, which is linked to the data points by vertical lines. (Online version in colour.)

species. Body length shows a somewhat more conserved pattern of evolution (electronic supplementary material, figure S2).

(b) Selective environment

Twenty-six *Rediviva* species had 4029 interactions with 96 species of oil-secreting plants. The selective environment of each bee species consisted of between one and 24 (median = 6.5) oil-secreting plant species (figure 3; electronic supplementary material, figure S3). The average floral spur length of these species varied from 0 to 25 mm (electronic supplementary

Table 1. Multiple linear regression estimates for the relationship between *Rediviva* leg length evolution, body size evolution and change in the selective environment imposed by the spurs of the host plants. Measurements are mm. Multiple R-squared $= 0.8634$, adjusted R-squared $= 0.8463$, $p < 0.0001$, d.f. $= 16$ (figure 5).

	estimated slope	s.e.	t-value	p	sum of squares (% variance explained)
environment change	0.4662	0.0603	7.731	<0.0001	334.98 (80%)
body evolution	0.6391	0.2321	2.754	0.0141	27.18 (6%)

material, table S2), and was frequently mismatched with the leg length of the *Rediviva* species that was captured on it. Nevertheless, an overall trait-environment correlation was evident (figure 4; electronic supplementary material, figure S4).

(c) Quantifying evolutionary change in traits and environment

Eighteen contrasts were calculated for each of the three variables (electronic supplementary material, figure S5). The three largest contrasts in leg length (12.7, 9.5 and 5.6 mm) occurred in very recently diverged sister species pairs. It was not necessary to transform the contrasts because they did not increase in magnitude with time since divergence. The regression of the absolute value of contrasts against the square root of the sum of their branch lengths was not significant and in two cases had a negative slope (leg $R^2 = 0.10$, $p = 0.11$, slope $= -1.00$; body $R^2 = 0.02$, $p = 0.27$, slope $= 0.30$; spur $R^2 = 0.10$, $p = 0.11$, slope $= -1.82$). Untransformed contrasts have the benefit of a clear meaning, which can be expressed in the original units of measurement (mm).

(d) Correlation analysis

Evolutionary change in leg length was linearly related to evolution of body size and to change in the selective environment imposed by host plants (figure 5). Together these two variables accounted for 86% of the variance in leg length evolution. When change in the selective environment was added first, this alone explained 80% of the variance in leg evolution, with body size evolution contributing an additional 6% of uniquely explained variance. When body size evolution was added first, it explained 51% of the variance in leg evolution, with change in the selective environment contributing an additional 35%. The model overall was highly significant (table 1), and the residuals were normally distributed (Shapiro–Wilk statistic $= 0.96$, $p = 0.67$). When the analysis was repeated with a reduced dataset form which contrasts at internal nodes were excluded, change in the selective environment explained 96% of the variance in leg evolution, with body size evolution contributing an additional 0.08% of uniquely explained variance (electronic supplementary material, figure S6 and table S3).

4. Discussion

The unusually long legs of *Rediviva* bees have originated in the selective environment imposed by long-spurred flowers and thus fulfil one of the two requirements of adaptation. It remains to be shown that natural selection is the driving force [3]. In contrast with the large number of studies that focus on floral adaptation, this study is among a small number that test pollinator adaptation [4,41–43].

It seems paradoxical that some *Rediviva* species should evolve long legs when they visit plant species with both long and short spurs. This may occur if long legs are necessary for foraging on long-spurred flowers, but do not hinder foraging on short-spurred flowers [44]; or, if visits to short-spurred flowers are less frequent [45]. Our analysis supports the latter scenario by showing that leg length evolution correlates with change in floral spur length when spur length is weighted by interaction frequency.

This study complements microevolutionary studies of the match between traits and environments in *Rediviva*. Steiner and Whitehead [22,32] found that intraspecific variation in two *Rediviva* species was correlated with variation in the spur length of their host plants, and to a lesser extend with body size variation. In a third species, which collects oil only from plants that lack spurs, intraspecific variation in leg length was strongly correlated with body size. In the macroevolutionary analysis presented here, body size variation explained a small percentage of the remaining variation in leg length after variation in floral spur length had been accounted for (figure 5 and table 1).

The selective environment considered here consists of the traits of interacting species and this opens the interesting possibility that the environment may not only be the agent, but also the target of selection, leading to a race [46]. If co-evolution is co-adaptation [47], we propose that testing for pairwise coevolution is a four-step process. The first two steps would be to test for selection. Each trait must be shown to provide the selective environment that favours the other [4,48–50]. The third and fourth steps are to test for evolutionary change in each of the traits in response to change in the selective environment imposed by the interacting trait. Here we meet one of the latter requirements by demonstrating evolutionary change in leg length in response to change in floral spur length. A complementary study could use a *Diascia* phylogeny, and methods similar to those used here, to test whether evolutionary change in spur length occurs in response to change in the selective environment imposed by long-legged bees.

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