The evolution of signal–reward correlations in bee- and hummingbird-pollinated species of *Salvia*

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Within-individual variation in floral advertising and reward traits is a feature experienced by pollinators that visit different flowers of the same plant. Pollinators can use advertising traits to gather information about the quality and amount of rewards, leading to the evolution of signal–reward correlations. As long as plants differ in the reliability of their signals and pollinators base their foraging decisions on this information, natural selection should act on within-individual correlations between signals and rewards. Because birds and bees differ in their cognitive capabilities, and use different floral traits as signals, we tested the occurrence of adaptive divergence of the within-individual signal–reward correlations among *Salvia* species that are pollinated either by bees or by hummingbirds. They are expected to use different floral advertising traits: frontal traits in the case of bees and side traits in the case of hummingbirds. We confirmed this expectation as bee- and hummingbird-pollinated species differed in which specific traits are predominantly associated with nectar reward at the within-individual level. Our findings highlight the adaptive value of within-individual variation and covariation patterns, commonly disregarded as ‘environmental noise’, and are consistent with the hypothesis that pollinator-mediated selection affects the correlation pattern among floral traits.

1. Introduction

The functional value of advertising and reward traits expressed by flowers has historically been the focus of scientific research. Both advertisement traits and the amount and quality of floral rewards show significant levels of within-individual variation [1–7]. Although part of this variation in rewards may result from previous visits by pollinators, recent evidence indicates that a large portion of within-individual variance in floral traits is constitutive of the plant phenotype [5–7]. Hence, multi-flowering species are expected to express a complex trait resulting from the simultaneous variation of advertisement and reward traits. Although natural selection has been predicted to favour the association between advertising and reward traits [8–10], there have been few attempts to experimentally demonstrate the functional value of trait combinations [11]. Whereas a number of studies have addressed the multivariate structure of functional floral traits within populations [12–14], the functional value of the within-individual covariation in advertising and reward traits has rarely been studied. Thus, the expected within-individual relationship between floral signals and reward is still an untested assumption.

When a pollinator visits a sequence of flowers from the same plant, it experiences a continuum of variation in advertising traits and in the amount and quality of rewards (ranging from full rewarding to deceptive flowers [4]). Pollinators, in turn, are able to gather information about signal–reward correlation through associative learning [15,16], and they are able to combine this information with...
previous knowledge to assess patch quality [17]. As long as plants differ in the reliability of their signals and the pollinators base their foraging decisions on this information, natural selection is expected to act on within-individual correlation between signals and rewards. Accordingly, recent evidence has demonstrated that pollinator-mediated selection favours high levels of signal accuracy, increasing floral honesty in *Turnera ulmifolia* [18]. In addition, theoretical models have suggested that in repeated consumer–resource interactions, the ability of the consumer to verify the accuracy of the signal after it has responded to it (i.e. signal verification) can promote and maintain reliable signalling [19]. However, no studies are available assessing the evolution of signal–reward within-individual correlations using a macroevolutionary approach.

Given that pollinator species vary in their cognitive capabilities, foraging patterns and energetic requirements, they use different signal–reward combinations. For example, pollinators that must land on the flowers to forage (e.g. bees, flies and wasps) use mainly frontal advertisement floral traits, whereas pollinators that are able to hover and easily change their flight direction (e.g. hummingbirds, bats and hawkmoths) are expected to use side advertisement floral traits [20]. Thus, depending on their foraging abilities, pollinators should target different advertisement traits and hence their correlation with rewarding traits. An ideal system to test this hypothesis is the clade of Neotropical *Salvia* species, which has distinct bee and hummingbird pollination syndromes. Bee pollination is ancestral in this group, and evolution towards ornithophily includes modifications in length, colour and shape of the corolla, nectar guides, nectar quantity and composition, and in the lever mechanism of anthers [21]. During the evolutionary transition from bee to hummingbird pollination, the signal–reward correlations should have simultaneously experienced adaptive changes. In this study, we examined whether the evolution of ornithophily in *Salvia* subgenus *Calosphace* involved a change from frontally based to lateral advertising traits. We predicted greater within-individual covariation between the lateral area of the corolla and the amount of reward in hummingbird-pollinated species. By contrast, in bee-pollinated species, within-individual covariation between frontal advertising traits and rewards should have been selected.

Using comparative phylogenetic analyses, we tested the hypothesis of adaptive divergence of the signal–reward correlation between pollination syndromes. Following Hansen [22] and Butler & King [23], we discriminated among alternative neutral and adaptive hypotheses concerning the evolution of this quantitative trait. While neutral models assume a Brownian motion (BM) process of trait evolution, adaptive hypotheses use an Ornstein–Uhlenbeck (OU) process to model the evolution of one or more continuous traits. Each OU hypothesis predicts a different distribution of adaptive optima on the branches of a phylogeny corresponding to selection regimes that currently and historically operate on the study system. To this end, we estimated within-individual signal–reward correlation in a group of *Salvia* species including bee- and hummingbird-pollinated species, and built a molecular phylogeny as the raw material for the construction of different adaptive scenarios. If bird- and bee-pollinated species of *Salvia* evolved different patterns of signal–reward correlation, we predict that an OU hypothesis corresponding to two adaptive optima should better explain the variation in the signal–reward correlation among species than alternative adaptive or neutral explanations.

2. Material and methods

(a) Study species

*Salvia subgenus Calosphace* is a monophyletic group of Neotropical plants [24] distributed from the United States to Chile and Argentina. Their flowers present complex bilabiate or tubular floral architectures and contrasting pollination syndromes, with most of them pollinated by bees, and about one-third pollinated by hummingbirds [21]. We sampled 18 *Salvia* species from natural populations in Mexico and Argentina (figure 1a,b; electronic supplementary material, table S1): S. *atrocyanus*, S. *calephos*, S. *cinnabarina*, S. *cuspidata* ssp. *gilletii*, S. *elegans*, S. *fulgens*, S. *guaranitica*, S. *iodantha*, S. *Iwandeloukoles*, S. *Longispicata*, S. *mexicana*, S. *misella*, S. *pallida*, S. *personata*, S. *pohstachya*, S. *purpurea*, S. *stachydifolia* and S. *thyrsiflora*. The main type of pollinator of each species was determined from literature [21,25,26] and confirmed with field observations for three of them (electronic supplementary material, table S2). Eight species (S. *atrocyanus*, S. *cinnabarina*, S. *elegans*, S. *fulgens*, S. *guaranitica*, S. *iodantha*, S. *mexicana* and S. *purpurea*) were mainly pollinated by hummingbirds, while the rest of the species were pollinated by bees.

*Salvia mexicana* and *S. purpurea* are highly variable species [21], and can be pollinated by both bees and hummingbirds. Our field observations, however, confirmed that hummingbirds were the main pollinators of *S. mexicana* at our study site (electronic supplementary material, table S2). In the case of *S. purpurea*, the flowers from our study site presented long corolla tubes and comparatively short and flexible lower lips, features that allow birds to access the nectar, excluding bees [21].

(b) Measured traits

We sampled 14–56 individuals per species and 3–15 flowers per individual (electronic supplementary material, table S1). For nectar measurements, plants were bagged at noon, and nectar volume and sugar concentration were recorded in the following day in freshly opened flowers using 1 or 5 µl micro-caps and
temperature-compensated hand refractometers (American Optical 10431). These variables were used to calculate nectar sugar content. Because flowers can accumulate nectar during the measuring period, we recorded the time at which each measurement was taken, and used the residuals from the regression between sugar content and time in the subsequent analyses. We took photographs of the side and front view of the same flowers used for nectar measurements along with a reference scale, using a Nikon D50 digital camera. Smaller flowers were photographed from closer distances, to ensure that flowers of different sizes occupied approximately the same relative area in the resulting photographs. For each photograph, we used the reference scale to transform the area measurements from pixels to square millimetres, following the instructions of UTHSCSA Image Tool v. 3.00 software. For each flower, we measured the frontal area of the lower lip and the lateral area of the corolla tube (figure 1c).

(c) Signal–reward correlation

When a research question involves assessing the joint change in the expression of two or more phenotypic traits, the sources of covariances can be distinguished. Because we were interested in the within-individual covariation of traits, we wanted to disentangle the within- and among-individual sources of covariation. Within-individual covariances are expected to be targets of pollinator-mediated selection, because pollinators can assess the variability of advertising and reward traits when they visit a sequence of flowers of the same plant. Hence pollinators can verify the reliability of plant signalling, and they can decide to leave or to continue foraging in a given plant based on this information [19]. By contrast, among-individual covariances are based on individual means, and thus they are statistical constructs that characterize populations, not individual phenotypes.

Disentangling within- versus among-individual sources of phenotypic variation requires the estimation of variance components, a task usually accomplished in ecological studies using mixed models [27]. We followed Dingemanse & Dochtermann [28] to disentangle the correlations between signals and reward from two hierarchical levels (i.e. within and among individuals). To this aim, we used Bayesian generalized linear mixed models (GLMM) with a bivariate response using the MCMCGlmm package [29] of R software [30]. In these models, the reward (nectar sugar content) and signals (area of lower lip or area of corolla tube) were treated as a joint response, and individual identity was introduced as a random effect. The posterior distributions of two variance–covariance matrices were estimated from this model: (a) the variance–covariance matrix for the individual random effect, in which the diagonal corresponds to the variances of consistent individual effects in rewards and signals, and the off-diagonal value is the covariance between them; and (b) the residual variance–covariance matrix, which estimates the equivalent variances and covariances within individuals. Using the latter matrix, the within-individual correlation between signal and reward was estimated as the Pearson correlation coefficient.

Bayesian GLMMs were run for 130 000 iterations, with a burn-in of 30 000, a thinning interval of 100 and flat gamma-inverse priors [28]. For each correlation, we obtained the 95% highest probability density (HPD) intervals. The posterior modes of the within-individual correlations were used in the comparative analyses. Two models were fitted per species: one where the diagonal corresponds to the variances of consistent individual effects in rewards and signals, and the off-diagonal value is the covariance between them; and (b) the residual variance–covariance matrix, which estimates the equivalent variances and covariances within individuals. Using the latter matrix, the within-individual correlation between signal and reward was estimated as the Pearson correlation coefficient.

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(d) Phylogenetic analysis

The estimation of a phylogeny with branch lengths is required to test whether a character has evolved under drift or under selection with one or more adaptive optima. With this aim, we compiled from NCBI GenBank database cpDNA sequences (the intergenic spacer psbA-trnH and the fragment including the intron and second exon of trnL gene and the intergenic spacer trnL-trnF) from 70 taxa, involving 53 species from Salvia subgenus Calosphaic and 17 outgroups. In addition, we generated the sequences for those species for which we measured the morphological and nectar traits, except for S. longispicata. PCRs were carried out using Qiagen multiplex PCR kits (Qiagen) with initial denaturation for 15 min at 94°C, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 1 min, and a final extension for 1 min. Sequences were aligned using MUSCLE [31], and verified by eye using StAView [32]. Insertion/deletion mutations (indels) were coded manually following the simple method of Simmons & Ochoterena [33]. We estimated the phylogeny using a Bayesian approach as implemented in the program BEAST v. 1.7.5 [34], using a GTR + I + I substitution model and non-informative priors for all parameters. Three independent Monte Carlo Markov chains (MCMCs) were run for 50 million generations. Trees were saved every 1000 generations. Log files from each run were imported into TRACER v. 1.4.1, to examine effective sample sizes (ESS) and stationarity. This test suggested that the analysis had reached stationarity within 50 million generations and the ESS of all parameters exceeded 200. Tree files from individual runs were combined using LOGCOMBINER v. 1.5.3 after removing 5000 trees from each sample. The maximum-clade-credibility tree topology was estimated from the posterior distribution of the trees (electronic supplementary material, figure S1). This ultrametric tree with branch lengths expressed in units of substitutions per site was pruned to include only the 18 taxa for which morphological and nectar data were collected. The corresponding internal branches were removed along with external branches during pruning.

(e) Comparative analyses

Before modelling the evolution of signal–reward correlations, we employ phylogenetic generalized least squares (PGLS) to characterize differences among pollination syndromes in the mean values of reward (nectar sugar content), signals (area of the corolla lower lip and area of corolla tube) and the predominance or not of frontal over lateral signals (i.e. area of the corolla lower lip minus area of corolla tube).

We modelled the evolution of within-individual signal–reward correlations under a BM process (which describes the neutral evolution of a quantitative trait) and under different OU processes (which describe different adaptive hypotheses corresponding to different selective regimes [22]) using the OUCH package for R [23]. This method uses maximum likelihood to estimate the strength of random drift (α, the only parameter in BM models), the strength of selection (β) and one or more values of
Phylogenetic uncertainty can affect our results, in particular because different topologies can result in different ancestral selective regimens on within-individual signal–reward correlations were tested: in model 1, within-individual signal–reward correlations evolved following a pure drift (BM) process. In model 2, these correlations evolved under a single selective scenario following an OU process with global evolutionary optimality (i.e. all species are attracted to a single adaptive peak in phenotypic space, but random drift simultaneously causes fluctuation around the optimum). In model 3, within-individual signal–reward correlations evolved under selection following an OU process towards two adaptive optima corresponding to different pollination syndromes, melitophily and ornithophily. Ancestral pollination syndromes were reconstructed by parsimony using the MPR function of the ape package in R [35], in order to estimate the selective regimes that historically operated on this group of plants. The 70 species of Calosphaeraceae included in the phylogeny as well as S. californica (as an outgroup) were used for reconstruction of ancestral states (electronic supplementary material, figure S2). Then, this tree with estimated ancestral states was pruned to include only the 18 taxa under study. Finally, model 4 hypothesized that signal–reward correlations evolved under selection towards three adaptive optima. Here, ornithophilous species were divided into two groups based on flower architecture following Wester & Claßen-Bockhoff [21]: 'bilabiate' and 'tubular or intermediate'. Ancestral regime reconstruction was performed as described for model 3 (electronic supplementary material, figure S3). To compare the fit of the models, we used likelihood-ratio tests of each model against the BM model, the Akaike information criterion corrected for small sample size (AICc) and the Schwartz information criterion (SIC). We calculated 95% confidence intervals for the parameter estimates (σ, α and θ) of the best model using a parametric bootstrap with 10,000 simulations.

Phylogenetic uncertainty can affect our results, in particular because different topologies can result in different ancestral selective regime reconstructions. To explore the effect of this uncertainty, we repeated the entire process described above in 1000 trees sampled from the posterior distribution of phylogenetic trees [35] and compared the fit of the four evolutionary models. Thus, we obtained 1000 different log-likelihood, AICc and SIC values for each model, and used these values to examine the consistency of the best evolutionary model. Ancestral state reconstruction for some nodes can also give uncertain results, entailing a problem for the specification of OU models 3 and 4. Thus, we followed two alternative approaches to deal with this source of uncertainty in ancestral reconstruction: first, we arbitrarily assigned melitophily as the ancestral state of all the dubious nodes, because reversal from long to short flowers (i.e. from ornithophilous to melitophilous) is unlikely [36]; second, we randomly chose between ornithophilous and melitophilous, and assigned this arbitrary ancestral state to the uncertain node. Both procedures were applied to the comparison of evolutionary models in the 1000 trees sampled from the posterior distribution of phylogenetic trees. However, as they yielded almost identical results, we reported the results assuming that melitophilous is the ancestral state in all dubious nodes.

3. Results

Hummingbird-pollinated species had, on average (± s.e.), 1.743 ± 0.372 µg more sugar in the nectar than bee-pollinated species (t = 4.681, p = 0.0003). Hummingbird-pollinated species had also larger corolla tubes than bee-pollinated species (mean difference = 0.0777 ± 0.372 µm, t = 1.885, p = 0.0777). In all ornithophilous species except S. atrocyanea, the area of the corolla’s lower lip was smaller than the area of the corolla tube, while the opposite pattern was evident in melitophilous species (mean difference in preponderance of ornithophilous species was 0.0778 ± 0.1233 mm², t = 5.738, p < 0.0001). Descriptive statistics of species means, and within-individual,
Table 1. Performance of BM model of evolution and OU models with one, two or three adaptive optima for within-individual correlations between signals and reward. Best model is in italics. In, log likelihood; LRT, likelihood-ratio test for each model compared with the Brownian motion (BM) model; AICc, Akaike information criterion corrected for small sample size; SIC, Schwartz information criterion; d.f., model degrees of freedom.

<table>
<thead>
<tr>
<th>parameter</th>
<th>model 1 (BM)</th>
<th>model 2 (OU, global optima)</th>
<th>model 3 (OU, two optima)</th>
<th>model 4 (OU, three optima)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lnL</td>
<td>7.510</td>
<td>12.590</td>
<td>17.730</td>
<td>17.984</td>
</tr>
<tr>
<td>LRT (p)</td>
<td></td>
<td>10.160 (0.017)</td>
<td>20.493 (0.001)</td>
<td>20.948 (0.003)</td>
</tr>
<tr>
<td>AICc</td>
<td>−3.021</td>
<td>−3.847</td>
<td>−6.660</td>
<td>1.597</td>
</tr>
<tr>
<td>SIC</td>
<td>2.897</td>
<td>3.488</td>
<td>0.375</td>
<td>7.034</td>
</tr>
<tr>
<td>d.f.</td>
<td>5</td>
<td>8</td>
<td>10</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 2. Parameters for the OU model with two regimes, corresponding to pollination syndromes: the strength of random drift (\(\sigma\)), the strength of selection (\(\alpha\)) and the values of adaptive optima (\(\theta\)). Ninety-five per cent CIs were obtained using parametric bootstrap. Parameters with a CI that does not include zero are in italics.

<table>
<thead>
<tr>
<th>parameter</th>
<th>within-individual correlation between lower lip area and reward</th>
<th>within-individual correlation between corolla tube area and reward</th>
<th>correlated evolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\alpha)</td>
<td>9.572 (2.259; 297.612)</td>
<td>6.067 (1.956; 422.093)</td>
<td>−3.198 (−215.923; 38.305)</td>
</tr>
<tr>
<td>(\sigma)</td>
<td>0.686 (0.018; 10.251)</td>
<td>0.427 (0.005; 14.000)</td>
<td>0.163 (−1.792; 4.112)</td>
</tr>
<tr>
<td>(\theta) melitophily</td>
<td>0.244 (0.130; 0.372)</td>
<td>0.154 (0.039; 0.271)</td>
<td></td>
</tr>
<tr>
<td>(\theta) ornitophily</td>
<td>0.148 (−0.001; 0.289)</td>
<td>0.313 (0.183; 0.477)</td>
<td></td>
</tr>
</tbody>
</table>

Discussion

It has been suggested that within-plant variation in floral trait expression could mask phenotypic differences among individuals, and hence could weaken selection pressures on floral evolution [1,3]. This expectation makes sense considering the evolution of average plant features: for instance, high within-plant variation in nectar could make it more difficult for a pollinator to differentiate among plants based on mean nectar production rates [1]. However, this rationale does not apply if the target of selection is within-individual variability itself. This variation may influence plant fitness by altering the diversity and composition of pollinator assemblages, modifying overall attractiveness of plants to risk-averse pollinators or affecting the rates of geitonogamous pollination [6]. Thus, if within-individual variation and covariation among traits have a genetic basis, the adaptive evolution of these attributes could be expected. This work supports the hypothesis that within-plant signal–reward correlations have followed an adaptive evolutionary pathway, because it fulfils the prediction that hummingbirds and bees select on different associations between advertising and reward traits, in accordance with their differential use of lateral and frontal floral traits as signals [20].
Alternatively, this evolutionary pattern could be a side effect of the modification of flower architecture during transitions from melitophily to ornithophily in Neotropical Salvia species. Because hovering pollinators like hummingbirds do not need a landing platform for foraging in flowers, evolutionary transitions to ornithophily often involve a reduction of the lower lip of the corolla [21,37,38]. This reduction may be achieved by decoupling the lower lip and other flower parts, and hence a relaxation in the correlation between the lower lip and nectar production rate. Our results, however, point to an inversion in the correlation patterns. Hummingbird-pollinated species not only have lower within-individual correlation between lower lip area and reward, but also show an increase in the correlation between corolla tube and nectar. Thus, our findings are consistent with the hypothesis of pollinator-mediated selection on within-individual correlations between different advertising traits and rewards. An open question is whether changes in pollination syndrome have driven further modifications in within-individual correlation patterns. In such cases, studies about phenotypic integration in modular organisms [12–14] should consider the within-individual source of variation in phenotypic traits.

Nectar production rate and flower length have undergone correlated evolution in hummingbird-pollinated species [39]. Although this correlation may be only a consequence of allometric relationships, this pattern is consistent with pollinator-mediated selection for larger flowers and high nectar production rate [39]. We emphasize the distinction between the correlated evolution of morphological and reward traits in flowers and the evolution of within-individual correlations. We did not test whether these traits experienced correlated evolution in Salvia. By contrast, we describe for the first time the macroevolution of within-individual correlations considered as complex phenotypes. Although there could be underlying causes such as developmental instability or reaction norms inside inflorescences [2,6], our results suggest that this complex phenotype could be a target of natural selection.

Our findings highlight the adaptive value of within-individual variation and covariation patterns, commonly disregarded as ‘environmental noise’. We consider that within-individual association between signals and rewards constitute the basis of a subtle strategy that ranges from faithfully signalling plants to deceptive ones, and both extremes can be present within a single plant population [18]. Hence, these traits could be the source of adaptive evolution of different signalling strategies in plants. Evidence for different adaptive optima corresponding to different pollinator groups gives support for the adaptive role of these traits and opens new avenues for the study of the evolution of signal honesty in plants. This hypothesis should be investigated using, for example, manipulative experiments [11,40] on contemporary populations of Salvia species that are pollinated by both bees and hummingbirds. Future research should also investigate the genetic basis of within-individual associations between floral signals and rewards, and the behavioural responses of pollinators to changes in these variables.

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