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Are nectar guide colour changes a reliable signal to pollinators that enhances reproductive success?

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Running title: Nectar guides and their changes
Abstract

Background: Ageing and post-pollination changes in floral colour occur widely in flowering plants, but it remains an open question as to whether or not colour changes in nectar guides are associated with the quantity of floral rewards that ultimately influence pollinator visitations and reproductive success.

Aims: To examine whether nectar guide changes should be considered as a reliable signal to pollinators and to assess the effects of nectar guide changes on reproductive success.

Methods: We studied the process and adaptive value of colour changes in the nectar guides of *Arnebia szechenyi* whose flowers typically display conspicuous nectar guides at the onset of anthesis, after which they begin to fade, and disappear completely on the second day.

Results: Changes in nectar guide colour in *A. szechenyi* were intrinsic and age-dependent, although pollination somewhat accelerated the change. By the time that the nectar guides disappeared completely, floral rewards were reduced almost to zero. Artificial removal of nectar guides decreased both fruit set and pollen export. Flowers without nectar guides do not appear to increase the overall attractiveness of the plants.

Conclusions: Nectar guides and their changes represent reliable signals to pollinators and enhance both male and female reproductive success.

Keywords: *Arnebia szechenyi*; floral display size; nectar guide; non-rewarding flowers; reproductive success
Introduction

Communication between plants and their pollinators involves a complex interplay of different sensory modalities (Raguso 2004; Willmer 2011). Pollinators may choose which flowers to visit by means of ‘honest’ advertisements or other traits correlated with the quantity or quality of rewards (Lunau 1992; Armbruster 2005). One of the most obvious examples of this reward-advertisement correlation (‘honest signalling’) by flowering plants is the case of post-pollination changes in floral colours that occur when the pollinator rewards are no longer produced (Delph and Lively 1989, Weiss 1991, 1995; Lunau 1996; Ruxton and Schaefer, 2016). ‘Unrewarding’ flowers are sometimes maintained in certain species, presumably because they increase the floral display size, and therefore contribute to pollinator attraction from a distance (Gori 1983, 1989; Oberrath and Böhning-Gaese 1999). Such floral colour changes may guide experienced floral visitors to fresh, rewarding/unpollinated flowers and increase the foraging efficiency of pollinators (Gori 1983, 1989; Casper and La Pine 1984; Kruijer 1987).

Among the various colour patterns of the corolla, the presence of so-called floral nectar guides is traditionally considered to indicate the location of floral rewards (e.g. nectar and pollen) to pollinators (Kevan 1972; Lunau 1992). Nectar guides in flowers are taxonomically widespread (Weiss 1995), and insects show biased attraction towards nectar guides when perceived at a close range (Waser and Price 1983, 1985; Dafni and Giurfa 1999), making these ‘signposts’ an important factor that affects which flowers the pollinators choose to visit (Hansen et al. 2011). However, the dynamics of nectar guide changes, in particular the extent to which their colour intensity is positively correlated to the amount of floral rewards available during a flower’s lifespan, and the effects of nectar guide changes on male and female fitness
of plants, remain poorly understood. This particular aspect is important in light of the ‘honest signalling’ hypothesis as it determines whether or not colour changes in nectar guides should be considered as reliable signals to pollinators.

In *Arnebia szechennyi* Kanitz (Boraginaceae), a perennial native herb of China, the bright yellow freshly opened flowers present five contrasting black patches on the corolla lobes, which we considered *a priori* as nectar guides (Figure 1). These contrasting patches begin to fade on the second day after flower opening, and have disappeared completely by the afternoon (Figure 1). Flowers without nectar guides are maintained for about two more days before wilting.

In this study, we investigated the dynamics and the adaptive value of the colour changes in the nectar guides in this species. Our objectives were: 1) to examine whether or not nectar guide changes were intrinsic and/or induced by pollination, and also whether they were correlated reliably with the amount of nectar and pollen rewards; 2) to assess the effects of nectar guides and their changes on male and female reproductive success, and 3) to examine the effects of floral display size on pollinator visitation so as to evaluate the role of flowers with faded nectar guides.

**Materials and methods**

*Study plant and site*

*Arnebia szechennyi* (Boraginaceae), typically inhabits sunny mountain slopes along the Yellow River in northern China. Our study was carried out in Xunhua County, Qinghai province, which is a typical habitat with a typical range of pollinators, from May to June, 2012 and 2014. *A. szechennyi* is characterised by distyly and heteromorphic self-incompatibility (Zhang et al., 2014), and thus pollinators are required for seed production. The anthesis lasts from May to July and flower longevity is 3-4 days. In
open flowers, anther dehiscence and stigma receptivity to pollen occur concurrently. The flower is salverform, around 1 cm in diameter, with a narrow corolla tube 0.95-1.28 cm long; the bicarpellate ovary has four chambers, each with a single ovule. Each chamber develops into an indehiscent nutlet, so that fruits consist of 0–4 one-seeded nutlets. For convenience of statistical analysis, we considered each nutlet as one seed in a flower (seed set equals number of nutlets per flower), and any flower that set at least one nutlet was considered as fruit setting. Our observations indicate that the most efficient pollinators were a hitherto undescribed long-proboscid bee-fly (Bombylius sp., Diptera Bombylidae), which visited the flowers for nectar, and a small mining bee (Nomiapis femoralis Palllas, Hymenoptera Halictidae), which visited A. szechenyi for pollen. Zhang et al. (2014) reported no differences in visitation rate per flower per hour between the two main pollinators and the two distylos morphs. The visitation rate per flower per hour of Bombylius sp. in long- and short-styled morphs was 0.009 ± 0.002 and 0.013 ± 0.006, respectively, while the visitation rate per flower per hour of Nomiia femoralis Walker in long- and short-styled morphs was 0.008 ± 0.002 and 0.012 ± 0.004, respectively.

Process of colour change in nectar guides
To test whether pollination triggered changes in the nectar guide colour patterns in A. szechenyi, 30 long- and 30 short-styled flower buds (one bud per plant) were selected and netted to exclude any insect visitations. Then 15 flowers of each morph were hand pollinated immediately after they opened using fresh pollen from a cross-compatible morph. In the short-style morph the stigma is located within the narrow corolla tube, and so a small slit was cut in the middle of the tube to effect pollination.
We assessed the phenological stages of the 60 flowers and their nectar guide colour
changes every three hours since they opened, including flowers with clearly visible nectar guides, fading nectar guides and disappeared nectar guides. The longevity (days = total hours/24) of the nectar guides and flowers was recorded until all the flowers had wilted. Two-way ANOVA analysis was employed to evaluate effects of flower morph and treatment on the longevity of the nectar guides and flowers, respectively. Differences of the longevity of the nectar guides and flowers between treatments were evaluated by \( t \)-test.

**Nectar production and pollen availability**

The nectar production of flowers of different ages, defined by the age of nectar guides as: early (flowers with clearly visible nectar guides), middle (flowers with fading nectar guides) and late (flowers without visible nectar guides), was measured using glass capillaries with an inner diameter of 0.5 mm and a digital caliper. According to our observations, there was almost no pollinator visitation before 08:00 h; therefore, at 06:00 h, before pollinator visitation, 20 flowers of different stages (early, middle and late) were selected randomly for each morph, and the nectar amount in the flowers was measured. To measure the changes in pollen availability, the five anthers of a further 20 flowers of different stages were randomly collected, from each morph, in separate centrifuge tubes and fixed in 2 ml FAA solution (formalin: acetic acid: 70% ethanol at a ratio of 5:5:90 by volume, with a drop of detergent for full suspension; Dafni et al. 2005) to determine the number of pollen grains remaining in the anthers. After being thoroughly shaken to dislodge the pollen grains, the anther tissue was removed. The number of pollen grains in each flower was calculated based on counts of the number of grains in ten replicates of 10 μl from each pollen suspension (droplet on a slide and observed by microscope). Two-way ANOVA
analysis was employed to evaluate effects of flower morph and nectar guide stage on nectar production and pollen availability, respectively.

*Effects of nectar guide changes on reproductive success*

The effects of the nectar guides on reproductive success were examined by artificial marking of the nectar guides with two different colours of permanent ink (Winsor & Newton, London, England). A total of 240 flower buds were selected from both long and short-styled plants (1:1), with each bud coming from a different plant. As the flowers opened, each was subjected to one of three treatments: (1) all the nectar guides were marked with permanent black ink to maintain them, (2) all the nectar guides were marked with permanent yellow ink, effectively erasing them, (3) the flowers were left unmarked as control. When the flowers began to wilt, half in each group were randomly collected to measure the number of pollen grains remaining in the anthers; the remaining flowers in each group were left so that we could evaluate the fruit set and seed set when fruits were mature. To measure the average number of total pollen grains in the anthers of unvisited flowers, a further ten flower buds from each morph were randomly collected. In the laboratory, we split each anther and suspended all the pollen grains in 5 ml of water with a drop of detergent for full suspension (Dafni et al. 2005). The number of pollen grains in each flower was measured with the method as before. The number of pollen grains exported by the pollinators \( E_i \) was calculated by the difference value between the average number of total pollen grains in the anthers of unvisited flowers \( T \) and the number of pollen grains remaining in the anthers of treated flowers \( R_i \): 

\[
E_i = T - R_i.
\]

U-test was used to evaluate the effects of nectar guide changes on fruit set. As the sample size < 30, we conducted continuous correction in the test. One-way
ANOVA and Post hoc-LSD were used to evaluate the effects of nectar guide changes on seed set and pollen export. The numbers for seed set included only counts from fruits with at least one seed because it makes no sense to consider seed set of aborted fruits and the zero data would underestimate the results.

To investigate whether the nectar guide colour treatments had changed the spectrum of petal colour reflection, we measured the relative reflectance of *A. szechenyi* flowers in unmarked, black ink marked, and yellow ink marked samples (see Supplementary information). Then, we converted the raw reflectance spectra data into the blowfly colour vision model (Troje 1993) and individual loci in the bee colour hexagon (Chittka 1992) as proxys to the visual system of the long-tongued bee-fly and *Nomiapis femoralis*, respectively (see Supplementary information). In addition, the volatile constituents of the black and yellow inks were analysed using gas chromatograph-mass spectrometer and the sample amount of inks for testing was ten times that used to mark the flowers in the field.

*Effects of floral display size on pollinator visitations*

To determine the effects of floral display size on pollinators, we randomly selected plants under natural conditions and counted the number of opened flowers with and without nectar guides on each plant. Then, we observed these plants and recorded the numbers of the attracted pollinators (hovering by plants but did not contact the flowers) and their effective visits to plants (landing on flowers and contacting the stigmas/anthers), and calculated the visitation rate per flower. In total, 3,027 flowers (1,170 flowers with nectar guides, 38.6%) on 58 plants were observed for a total period of 60 hours. The responses of the pollinators to plants with different floral display size were evaluated by the relationships between the floral display size and
the number of attracted pollinators per plant per hour, the number of visits per plant per hour, and the visits per flower per hour. These relationships were examined using Poisson regression, with all independent and dependent variables rounded down after multiplying by 10 to meet the assumptions of Poisson regression.

Results

Process of colour change in nectar guides

No significant differences were found between floral morphs in the longevity of the nectar guides ($F_{1,56} = 0.07, P = 0.80, n = 60$) and the longevity of flowers ($F_{1,56} = 0.02, P = 0.89, n = 60$) in any of the treatments applied and we therefore pooled the data for long- and short-styled flowers. For the netted flowers which were unpollinated, the nectar guides began to fade on the second day after the flowers opened and the longevity of the nectar guides was about $1.23 \pm 0.04$ days ($n = 30$, mean $\pm$ S.E., the same in the following), indicating that nectar guide change is an intrinsic trait in $A. $\text{szechewii}$. However, the longevity of the nectar guides was significantly lower at $0.99 \pm 0.01$ days ($n = 30$) ($t_{58} = 6.92, P < 0.001$) in the netted flowers which were subjected to hand pollination. No significant difference was found in floral longevity between hand pollinated and unpollinated flowers ($4.06 \pm 0.14$ vs $3.69 \pm 0.17$ days; $t_{58} = 1.68, P = 0.10, n = 60$).

Nectar production and pollen availability

As the nectar guides faded, the volume of nectar per flower decreased significantly compared to the flowers with visible nectar guides ($F_{2,114} = 17.65, P < 0.001, n = 120$), and almost to zero when the nectar guides had disappeared (Figure 2A). No differences were observed for nectar yield between floral morphs ($F_{1,114} = 1.93, P =$
0.17, n = 120). In addition, along with the fading of the nectar guides, the number of pollen grains remaining in the flowers also decreased significantly ($F_{2,114} = 94.13, P < 0.001, n = 120$) (Figure 2B), and significant differences of pollen availability were found between floral morphs ($F_{1,114} = 247.86, P < 0.001, n = 120$).

**Effects of nectar guide changes on reproductive success**

Our results on the average reflectance of the natural and artificially marked flowers suggested that pollinators cannot discriminate between the natural black and black ink (Figure 3A) and between the natural yellow and yellow ink (Figure 3B) (detailed in the Supplementary information). Moreover, in the volatile constituents test, there were no obvious difference in the types of chemical constituents between the black- and yellow ink as observed in the peaks from the Gas Chromatography-Mass Spectrometer (data not shown). In flowers with black ink covering and prolonging the nectar guides, fruit-set (i.e. flowers setting at least one nutlet) was significantly higher than in unmarked flowers of both morphs (long-style: $u = 10$, $P < 0.01$; short-style: $u = 12.5$, $P < 0.01$); however, seed set (i.e. number of nutlets per flower) and pollen export were not enhanced (Figure 4A,B). When the nectar guides of newly opened flowers were marked with permanent yellow ink, thus effectively erasing them, the fruit-set (long-style: $u = 9.52$, $P < 0.01$; short-style: $u = 2.78$, $P < 0.01$) and number of exported pollen grains (long-style: $LSD, P < 0.01$; short-style: $LSD, P < 0.01$) were reduced significantly in both morphs, but there were no significant differences in seed set (Figure 4A,B). The artificial maintenance of nectar guides increased fruit yield and their removal decreased fruit yield and pollen export of *A. szechenyi*.

**Effects of floral display size on pollinator visitations**
Pollinators showed a significant preference ($\chi^2 = 246.3, P < 0.001$) for flowers with nectar guides in that only a low proportion (4.4%, 13 out of 295 visits) of visitations occurred on flowers without nectar guides, and most of these visits (92.3%, 12 out of 13 visits) were by solitary bees. No significant difference was found in visitation rate (visits/flower/h) between long- and short-styled flowers with nectar guides by the long-proboscid bee-flies ($t_{46} = 0.51, P = 0.61$) or by the solitary bees ($t_{46} = 1.06, P = 0.30$), so we pooled the data for long- and short-styled flowers. The relationship between the number of open flowers per plant and number of attracted pollinators per plant per hour (Figure 5A,B) and visits per plant per hour (Figure 5C,D) was positive and significant ($P < 0.05$) for both the long-proboscid bee-flies and the solitary bees, but was not significant between the number of open flowers per plant and visits per flower per hour (Figure 5E,F) for the two kinds of pollinators.

**Discussion**

In *A. szechenyi*, hand pollination somewhat accelerated the disappearance of nectar guides, whilst flowers that were netted to exclude pollinators also showed a nectar guide colour change, implying that this colour change is also age-dependent. Along with the fading of nectar guides, both nectar production and pollen availability decreased significantly. In addition, both of the two types of pollinators of *A. szechenyi* significantly preferred flowers with nectar guides over older flowers, which indicates that pollinators can consistently discriminate between freshly opened, rewarding flowers and older flowers providing less to no rewards, as reported in *Fuchsia excorticata* (Forst. & Forst. f.) L. f. by Delph and Lively (1989). Our results illustrate that the change in the conspicuousness of the nectar guides represents a reliable or ‘honest’ quantitative indicator to pollinators of floral rewards in *A.*
However, one caveat is needed: it is possible that pollinators also use a cue other than colour (for instance scent) to distinguish between old and young flowers. As a result, future studies should check for differences in the floral volatiles as the flowers age.

The contrasting colour patterns and their changes not only allow the pollinators to transfer pollen more efficiently, but also direct pollinators to recently opened and therefore most likely unpollinated flowers, thus promoting greater levels of outcrossing and decreasing geitonogamy (Casper and La Pine 1984). When the pollinator first arrives at a plant, it potentially carries pollen from a different conspecific plant. Thus, a reliable signal might be particularly valuable when it directs the pollinator, so that the first few visits of the pollinator enable reproductively active flowers to exploit this outcrossing pollen (Ruxton and Schaefer 2016). This suggests that nectar guides might improve the male and/or female fitness of plant species (Medel et al. 2003; Schaefer et al. 2004; Hansen et al. 2011; Leonard and Papaj 2011) and therefore be under selection through male and/or female fitness components (Ashman and Morgan 2004; Pohl et al. 2006; Hansen et al. 2011; Zhang et al. 2012). In general, selection through the male function should be stronger than through female function when seed production is limited by available maternal resources (resource limitation), and the siring success is limited by mating opportunities (Bateman 1948). However, the opposite should prevail when seed production is limited by mating opportunities (pollen limitation) (Harder and Aizen 2010). In our study species, fruit output seems to be pollen-limited since flowers pollinated manually with inter-morph pollen increased fruit set significantly compared to naturally pollinated flowers in *A. szechenyi* (Zhang et al. 2014). Our nectar guide colour treatments showed that obliteration of the nectar guides significantly reduced
the number of exported pollen grains and fruit set (Figure 4A,B), suggesting that selection of nectar guide may occur through both male and female function in our study species. The experimental maintenance of nectar guides with permanent black ink increased fruit set significantly (Figure 4A,B), indicating that the maintenance of the reliable signal for nectar reward might have encouraged the pollinators to forage these older flowers with permanent nectar guides (Hansen et al. 2011; Leonard and Papaj 2011). The reason why nectar guides are not naturally maintained for longer, given this improved female reproductive success, might be that the advantage of maintaining them was possibly in conflict with some other constraint (e.g. resource limitation). Although *A. szechenyi* is a perennial herb, they root in poor soil and bloom even hundreds of flowers concurrently on a single plant, and thus there might be resource limitation during anthesis. In addition, refer to the fruit set of the whole plant, the maintenance of the nectar guides may not be beneficial because the repeated visits of the flowers with nectar guides might decrease the foraging efficiency of pollinators and finally reduce the reproductive fitness of the whole plant. The number of remaining pollen grains in different nectar guide stages showed that flowers had been depleted of most pollen grains that could be removed by pollinators by the stage that the nectar guides disappeared, so that making the nectar guides permanent did not lead to more pollen export. We hypothesize that the reason why there was no significant difference in seed set between the artificial marking treatments might be that there is a pattern of fixed ovule abortion occurs in *A. szechenyi*. Although a single pollinator can deposit enough pollen to effect fertilisation of all four ovules, it is likely that, as in other boraginaceous species (Casper and Wiens, 1981; Casper and La Pine 1984), ovule abortion usually occurs and only one or two nutlets survive to fruit maturation. In *A. szechenyi*, even when manually pollinated, some flowers still failed
to set fruits (17.5%, 7 out of 40 flowers) and seed set was not increased significantly; all flowers that set fruits produced only 1–3 nutlets ($n = 33$, number of 1, 2, 3 nutlets $= 7:17:9$) (Zhang et al. 2014).

Although nectar guides disappeared completely on the second day after flower opening, the flowers retained their yellow display until they wilt on the fourth day after opening. Our observations of pollinators suggested that individual plants of *A. szechenyi* with more open flowers attracted more pollinators (Figure 5A,B) and received more visits on average (Figure 5C,D). However, very few pollinators visited flowers without nectar guides, and this was the case regardless of the number of open flowers on the plant. Moreover, when a plant had more open flowers, the visitation rate to flowers with nectar guides was not higher (Figure 5E,F). Therefore, the higher visitation rate to plants with more flowers was simply because there were more flowers with nectar guides for pollinators to visit. Thus, flowers without nectar guides did not enhance attraction to the plant overall. Rather, a bigger floral display included more flowers with nectar guides which were the ones the pollinators visited and consequently there were more pollinators on the plant. An adaptive function for the persistence of flowers without nectar guides was not identified. Our nectar guide colour treatments showed that some of these older flowers had not been pollinated until the nectar guides faded, so there is a potential for additional reproductive success through these older flowers because a low proportion of visits occurred on flowers without nectar guides. In addition, perhaps physiological constraints prevent the plants from losing senescent corollas quickly and the plants may recover translocatable nutrients from these older corollas (Casper and La Pine 1984). It is also possible that maintenance of floral display by retention of flowers without nectar guides is important in attracting insects to more isolated plants (Casper and La Pine
1984) but the interplant distances with which we worked may not have been great enough for this effect to be evident.

Conclusions

Our results illustrate that in *A. szechynyi* nectar guides improve fruit set and pollen export, and indicate the importance of selection through both male and female function in shaping floral traits and the evolution of this honest floral signal (Stanton et al. 1986; Dudash et al. 2011; Zhang et al. 2012). Furthermore, the pollinators make foraging decisions based on the number of flowers with nectar guides and not the total number of flowers on the plant. Our results contribute to the understanding on the evolution of reliable signalling by flowers to pollinators, and they highlight the need to investigate their temporal dynamics to reach a more thorough understanding of their role in mediating pollinator visitation rates and their effect on plant reproductive success.

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Disclosure statement

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**Supplemental data**

Detailed methods and results on reflectance spectra.

**Notes on contributors**

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References


Figure legends

Figure 1. Flowers of *Arnebia szechenyi* in different nectar guide stages, indicating the early stage with a complete nectar guide, middle stage with a fading nectar guide, and late stage when the nectar guide has disappeared.
Figure 2. Nectar production (A) and pollen availability (B) of *Arnebia szechenyi* in the three different nectar guide stages.
Figure 3. A. Loci of flowers with clearly visible nectar guides (grey squares) and flowers with nectar guides marked by black ink (dark squares) in the bee (hexagon) and fly (square) color space. B. Loci of flowers without nectar guides (yellow squares) and flowers with nectar guides marked by yellow ink (yellow triangles) in the bee (hexagon) and fly (square) color space.
Figure 4. Effects of different treatments on fruit set, seed set and exported pollen of long- (A) and short-styled (B) flowers of Arnebia szechynyi. Error bars indicate standard error, and different letters indicate the significant difference at $P = 0.05$ level.
Figure 5. Effects of the number of open flowers on the number of attracted pollinators per plant per hour for the long-proboscid bee-flies (A) and solitary bees (B), visits per plant per hour for the long-proboscid bee-flies (C) and solitary bees (D), and visits per flower (with nectar guide) per hour for the long-proboscid bee-flies (E) and solitary bees (F). The $p$-values here are testing for differences from a non-zero slope.
Supplemental data:

Detailed methods and results on reflectance spectra

Methods

To investigate whether the nectar guide color treatments had changed the discrete nature of petal color reflection, we used a portable spectrophotometer equipped with a PX-2 pulsed xenon lamp (Ocean Optics, Dunedin, FL, USA) to measure the relative reflectance (in %, 300–700 nm in 5-nm steps) of *A. szechenyi* flowers in 2014. The spectrophotometer was calibrated with a white standard. We then compared the reflectance spectra of flowers with clearly visible nectar guides and flowers with nectar guides marked by black ink, and the reflectance spectra of flowers without nectar guides and flowers with nectar guides marked by yellow ink. For each flower, we measured several times each color in order to get the average reflectance of the measurements.

We converted our reflectance spectra into the blowfly color vision model (Troje 1993) as a proxy to the visual system of the long-tongued flies identified as pollinators in this study, since the visual abilities of our study pollinator species are unknown to date. According to this model, flies discriminate spectral stimuli according to 4 different categories with boundaries at 400 and 515 nm (Troje 1993; Arnold et al. 2009), and the flies are considered to be able to discriminate among spectral stimuli that fall into different categories corresponding to different excitation levels of each type of receptors (Troje 1993; Arnold et al. 2009). Therefore, all stimuli within each category (quadrant) are considered indistinguishable from the flies’ perspective.

We used the spectral sensitivity functions of the honeybee (*Apis mellifera*) because they are representative of a wide taxonomic spectrum of higher
Hymenoptera, and they are largely consistent within the Apoidea (bees *sensu lato*) (Peitsch et al. 1992; Chittka and Kevan 2005). We converted the raw data (relative reflectance measurements) into individual loci in the bee color hexagon (Chittka 1992).

For the measurements of very dark colors from the bees’ perspective, we used a mid-grey adaptation background of 30 % reflectance across all wavelengths (300–700 nm) (Vereecken et al. 2013). All other measurements were performed using the reflectance spectra of the background vegetation. We assessed and quantified the color and achromatic contrasts by calculating (a) pairwise Euclidean distances between loci and (b) the mean Euclidean distance between the centroids for each set of ink/flower measurements in the bee color hexagon. The Euclidean distance between any two loci indicates the perceived color difference or contrast between the stimuli, and threshold values of hexagon units for color discrimination usually range between 0.062 (Dyer and Chittka 2004; Dyer et al. 2008) and 0.100 (Chittka et al. 1997) for bees.

**Results**

Our results on the average reflectance of the natural and artificial marked flowers suggested that pollinators cannot discriminate between the natural black and black ink and between the natural yellow and yellow ink (Fig. S1). In the bee color space, the distance between loci correlates to the discrimination capability of the spectral signals by bees. In the fly color space, spectral signals with loci in different quadrants are considered to be discriminated. The black signals show an extensive overlap near the center of the bee color space (mean color contrast between categories < 0.05), and they cluster in the same quadrant of the fly color space. These results suggest that the bees perceive both signals as achromatic (‘bee black’) and that neither the bees, nor
the flies are likely to be able to discriminate between the flowers with nectar guides and those marked with black ink. Similar results were also found for flowers marked by yellow ink, suggesting that both bees and flies cannot discriminate the flowers without nectar guides and those marked with yellow ink.

References


Figure S1. Spectrophotometric reflectance of the natural and marked flowers of *Arnebia szechenyi*. A. Dotted line: artificial black; solid line: natural black. B. Dotted line: artificial yellow; solid line: natural yellow.