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# Floral signposts: testing the significance of visual 'nectar guides' for pollinator behaviour and plant fitness

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Nectar guides, contrasting patterns on flowers that supposedly direct pollinators towards a concealed nectar reward, are taxonomically widespread. However, there have been few studies of their functional significance and effects on plant fitness. Most previous studies focused on pollinator behaviour and used artificial flowers in laboratory settings. We experimentally investigated the role of putative nectar guides in a natural system: the South African iris *Lapeirousia oreogena*, whose flowers have a clearly visible pattern of six white arrow-markings pointing towards the narrow entrance of the long corolla tube, and its sole pollinator, a long-proboscid nemestrinid fly. We painted over none, some or all of the white arrow-markings with ink that matched the colour of the corolla background. Although arrow-marking removal had little effect on the approaches by flies to flowers from a distance, it dramatically reduced the likelihood of proboscis insertion. Export of pollen dye analogue (an estimate of male fitness) was reduced to almost zero in flowers from which all nectar guides had been removed, and fruit set (a measure of female fitness) was also significantly reduced. Our results confirm that the markings on *L. oreogena* flowers serve as nectar guides and suggest that they are under strong selective maintenance through both male and female fitness components in this pollination system.

**Keywords:** pollination; nectar guide; pollinator behaviour; long-tongued fly; plant fitness

## 1. INTRODUCTION

> *'If now an insect, attracted by the beauty of the corolla, or by an agreeable odour, has gone to a flower, it will either forthwith perceive the nectar, or, if this is in a concealed place!, will not perceive it. In the latter case Nature comes to the rescue with the nectar-guide. This consists of one or several spots, lines, dots, or markings of another colour than that of the corolla as a whole, and consequently conspicuous against its lighter or darker tint. It is always placed just where the insects must creep in if they are to reach the nectar'*—[1]
> (translation by Ainsworth-Davies in Knuth 1906, p. 15).

Angiosperm flowers are often characterized by spatial cues which are thought to guide flower visitors towards rewards. The most widespread cues consist of converging lines or dots, or a marking around the corolla opening [2]. These 'nectar guides', first described by Sprengel [1], generally contrast strongly with the rest of the corolla. In some cases this contrast occurs mainly in the ultraviolet (UV) spectrum, making the guide patterns indistinct or invisible to humans [3,4]. Floral spatial cues that have been studied include contrasting patterns created by pollen, anthers or mimic anthers [5–7], flower shape [8], different petals with contrasting colours [9], scent [10], surface structure [11–13] and even acoustics [14]. In general, spatial cues in flowers are thought to improve the foraging efficiency of a pollinator by minimizing handling time, thus increasing the attractiveness of flowers, and they may also serve to physically align a pollinator, facilitating optimal pollen placement and subsequent deposition at another flower of the same species.

Despite the widespread taxonomical and geographical occurrence of visual line- and dot-shaped visual nectar guides (hereafter nectar guides), there has been surprisingly little documentation of their functional role in nature. Most nectar guides are relatively small compared with the rest of the perianth, and Sprengel [1] had already hypothesized that they primarily facilitate close-up orientation, rather than being long-distance attractants. However, early experiments found that nectar guides could affect attraction from a distance, as well as an insect's behaviour after it alights [15,16]—but these studies used very small flight cages and artificial models that did not mimic flowers very well. Later studies, using more natural flower models and larger flight spaces, have confirmed Sprengel's original hypothesis by demonstrating that nectar guides are primarily for close-up orientation [17–19].

The vast majority of experimental studies of nectar guides have focused on the behaviour of a limited range of insect taxa without considering their effects on plant fitness, and used artificial flowers that were often simple abstractions of general floral shapes and not based on features of real flowers (see electronic supplementary material, table S1; but see [9,17,20,21]). We are aware of only three studies that specifically addressed the relationship between putative nectar guides and plant fitness, all of which used *Mimulus luteus* and its pollinators as their model system and considered only female fitness. Two of these studies were correlational and illustrated how natural variation in nectar guide

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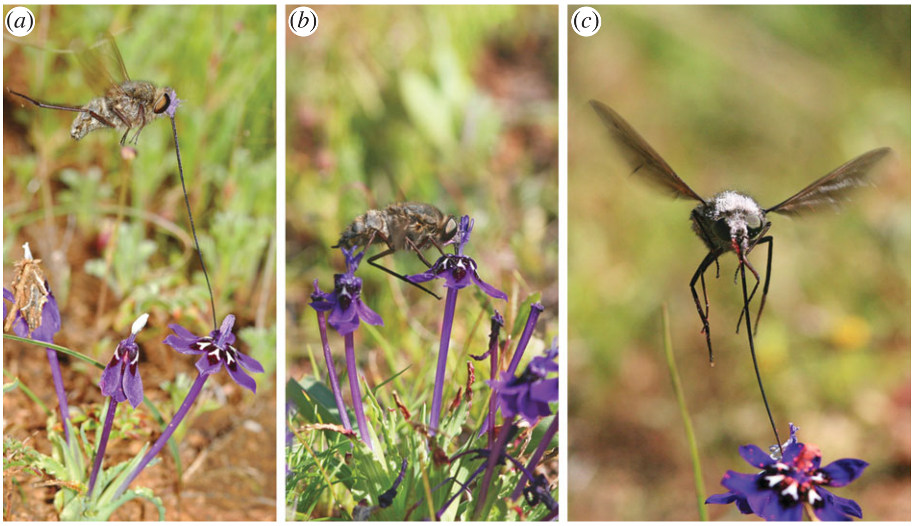

Figure 1. The long-proboscid nemestrinid fly *Prosoeca* sp. nov. foraging at flowers of the iris *Lapeirousia oreogena*. (*a*) Hovering fly navigating its proboscis towards the narrow perianth tube containing the nectar. (*b*) Feeding fly with proboscis fully inserted (partly visible through the thin-walled tube), pressing its head against the anthers and stigma of the flower. (*c*) Departure from a flower with pink UV-fluorescent dye applied to anthers; the fly has picked up dye grains on its frons.

morphology can affect plant female fitness, with pollinating insects and hummingbirds preferring different nectar-guide morphs [22], irrespective of the nectar-guide asymmetry [23]. In the third study, Pohl *et al.* [24] experimentally investigated the effect of flower herbivory using petal clipping that essentially removed the nectar guide; such manipulated flowers set fewer seeds than intact flowers.

Here we use simple, manipulative experiments with flowers in the field to assess the functions of visual floral guides with respect to both pollinator behaviour and plant fitness. Conspicuous visual floral patterns interpreted as nectar guides occur among southern African plants pollinated by long-proboscid flies [25]. These insects belong mainly to the Nemestrinidae (tangle-veined flies) and Tabanidae (horseflies) and have nectar-feeding proboscides up to 80 mm long in some species. Flowers of plant species pollinated by these insects have correspondingly long corolla tubes and are mainly in the Iridaceae, Orchidaceae and Geraniaceae [25–30]. These pollination systems are often highly specialized and recent evidence suggests that there has been widespread coevolution between the flies and their plant mutualists [31–33]. One of the most specialized systems involves the narrow endemic *Lapeirousia oreogena* (Iridaceae), which is pollinated solely by an as-yet undescribed long-proboscid fly, *Prosoeca* sp. nov. (Nemestrinidae) (figure 1; [28]). Flowers of this iris have a conspicuous pattern of six inward facing white arrow-markings around the mouth of the corolla (figure 1), which we hypothesized to be nectar guides.

We test two main predictions of this hypothesis, namely (i) that these arrow-markings function as close-up spatial cues for insertion of the proboscis, and (ii) that loss of these arrow-markings will negatively influence both male and female components of plant fitness.

## 2. MATERIAL AND METHODS

### (a) *Study system*

*Lapeirousia oreogena* is a narrow endemic, known only from the Nieuwoudtville district at the northern extremity of the Cape Floristic Region of South Africa. It is a small geophyte which has one to three open flowers at any one time (figure 1). Flowers generally remain open for 3–5 days and are protandrous, with stigmas fully extended and opening from day 2 or 3. They are unscented to the human nose and have a long, narrow perianth tube (4.5–6 cm long) containing a standing crop of *ca* 2.5–7.3 μl sucrose-dominated nectar with an average sugar concentration of 25.8 per cent [34]. The tepals form a radially symmetric, flat-topped display (figure 1). The outer half of each of the six tepals is purple, the inner half is black and the arrow-markings are white (figure 2). Reflectance spectra were measured with an Ocean Optics S2000 spectrophotometer and fibre-optic reflection probe (UV-Vis 400 μm), as described by Johnson & Andersson [35]. Goldblatt *et al.* [34] mentioned that *L. oreogena* had ‘been noted to set seed in greenhouses where insect activity was excluded’, concluding that it was partially autogamous. However, recent field studies have shown that *L. oreogena* depends on pollinator visits for seed production [36].

The undescribed long-proboscid fly, *Prosoeca* sp. nov. (Diptera: Nemestrinidae; figure 1) has a proboscis *ca* 40 mm in length [28,34]. The proboscis is stiff and non-retractable, so that its length defines the minimum distance between the fly’s body and the flower during initial manoeuvring to insert the proboscis into a floral tube. When foraging in a patch of *L. oreogena*, the fly approaches a flower in a fast, horizontal flight, some 5–10 cm above the plants, then hovers for 1–3 s above a flower (figure 1*a*), before it either inserts its proboscis or flies away. After inserting the tip of its proboscis into the perianth tube, the fly alights on the tepals, pushing the proboscis as far down the perianth tube as possible, causing the frons and the base of the proboscis to be pressed firmly against the anthers and/or stigma (figure 1*b*). Clearly visible patches of the purple pollen were observed on most foraging flies.

We conducted our study in a large population of several thousand plants in the Nieuwoudtville Wildflower Reserve (31°21’57.29″ S, 19°8’19.86″ E) in the succulent karoo region of South Africa. The plants occur in dense patches in open grassy areas between dolerite boulder outcrops. Experiments involving fly behaviour were conducted in

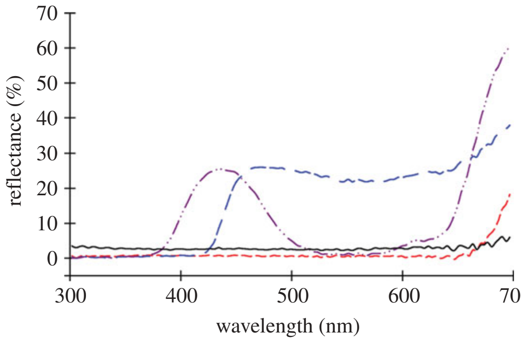

Figure 2. Reflectance spectra of *Lapeirousia oreogena* flower tepals, including the spectrum of a manipulated arrow-marking blotted out with black ink. Red dashed lines, arrow-marking plus ink; blue dashed lines, arrow-marking; purple dashed-dotted lines, tepal purple; black solid lines, tepal black.

August 2007 and those involving plant fitness measures were conducted in August–September 2008. All statistical analyses were carried out using PASW Statistics 18 (SPSS, Chicago, IL, USA).

### (b) *Arrow-marking manipulations*

For all experiments, we used a simple method of manipulation: we blotted out the required number of arrow-markings with permanent black marker ink. This resulted in a black colour with a reflectance very similar to the base colour of the tepal surrounding the arrows (figure 1 and see electronic supplementary material, figure S1). Control flowers in all experiments (i.e. flowers with all six arrow-markings present) had similar amounts of black marker ink applied to the black part of the tepals, without altering the arrow-markings.

### (c) *Pollinator behaviour*

All experiments involving fly behaviour were conducted on sunny or mostly sunny days, between 10.00 and 15.00 h, which is the peak activity period for the flies in the flower reserve.

#### (i) *Long-distance attraction*

To investigate the effects of arrow-markings on the long-distance attraction of flies, we selected 24 plants with one to three (1.8 ± 0.81 s.d.) fully open flowers, removing any partially open (opening or wilting) flowers. Plants were then randomly assigned to either the control group ($n = 12$ plants), or had all arrow-markings covered with black marker ink ($n = 12$ plants). All other flowers of *L. oreogena* plants within a 50 cm radius of the experimental plants were removed. Depending on weather conditions, each plant was observed for approximately 2.5 h, recording the number of flies that approached the plant (flying towards a plant and hovering just above flowers), and whether an approach resulted in subsequent probing of flowers. The observer could observe up to six plants simultaneously from one point. We analysed differences in the number of approaches using a generalized linear model with a Poisson error structure, while the proportion of approaches that resulted in probings was analysed using a generalized linear model with binomial error structure. Likelihood ratios were used for significance testing.

#### (ii) *Close-up choices*

To investigate effects of arrow-markings on close-up flower choice by a foraging fly, we selected flowering plants at least 1 m away from each other. On each plant, we removed open and wilting flowers until only two fully open flowers remained. In some cases, we could not find suitable single plants with two fully open flowers, in which case two closely neighbouring plants with a single, fully open flower each were considered as a pair. In both cases, one flower was randomly assigned as a control, whereas the other had a varying number of arrow-markings covered with black marker ink: (i) all six arrow-markings covered (40 plants), (ii) three arrow-markings covered, alternating between tepals (14 plants), or (iii) all but one arrow covered (15 plants). Depending on the weather conditions, each plant was observed for approximately 2.5 h, during which an observer recorded the flower of a pair chosen first by an approaching fly. The observer could observe between four and 10 pairs simultaneously from one point. We analysed these data as proportions of visits to flowers with all six arrow-markings in a generalized linear model with a binomial error structure. The significance of pairwise comparisons among mean proportions was assessed using the Dunn–Šidák procedure, while the significance of preference for a particular arrow-marking type was assessed by establishing whether the value of 0.5 (equal choice) lay outside the 95% confidence intervals for each mean proportion.

### (d) *Plant fitness*

#### (i) *Male fitness*

We investigated the effect of arrow removal on pollen export by using four colours of fluorescent dye powder (DayGlo Corp, OH, USA) as a pollen analogue. Prior to fly activity on four non-consecutive fair weather days, we haphazardly selected plants with fresh, fully open flowers, and applied liberal amounts of dye grains to the anthers by gently bending the flower and dipping the anthers into vials containing the dye powder. All other open flowers on these plants were removed. In the evenings, about an hour after dark, all open flowers within a 50 cm radius of the dyed flowers were checked for grains of fluorescent dye, using a hand-held UV-lamp. We dyed a total of 41 flowers; 21 with all six arrow-markings still present, and 20 with all arrow-markings painted black.

#### (ii) *Female fitness*

To investigate the effect of arrow-marking manipulation on female fitness, we compared fruit-set of flowers with and without these markings. On days with fair weather, we haphazardly selected a total of 76 plants that had an opening bud early in the morning and emasculated it before anther dehiscence and fly activity. All other open flowers and opening buds on each plant were removed. Flowers were assigned either as controls ($n = 38$ plants), or had all six arrow-markings painted ($n = 38$ plants). We harvested the fruits on 13 November 2008. Seeds were scored as viable if they were unambiguously larger than ovules. The significance of differences in the frequency of fruit set was assessed with Fisher's exact test, while the significance of differences in the mean number of seeds per fruit was assessed with a generalized linear model that incorporated a Poisson error structure and correction for overdispersion.

## 3. RESULTS

### (a) *Pollinator behaviour*

#### (i) *Long-distance attraction*

Flies approached plants with or without arrow-markings on their flowers with equal frequency (figure 3*b*; $\chi^2 = 0.202$, $p = 0.65$). However, manipulation of the arrow-markings significantly influenced whether an approach resulted in subsequent successful probing of the flower. All approaches

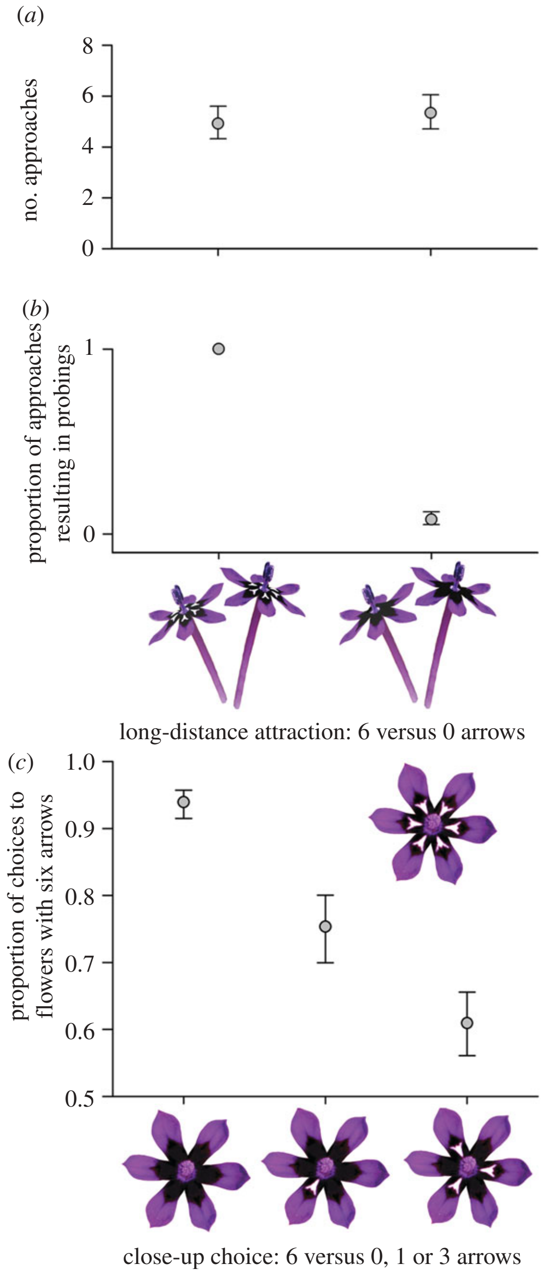

Figure 3. Long-distance attraction and close-up choice of *Prosoeca* sp. nov. at flowering plants of *Lapeirousia oreogena* with or without manipulated arrow-markings. (*a*) The number of long-distance approaches to plants having flowers with or without arrow-markings. (*b*) Proportion of approaches that resulted in subsequent probings. (*c*) Proportion of close-up first choices in favour of 'intact' flowers with all six arrow-markings present over manipulated flowers with zero, one or three arrow-markings present. Values shown are back-transformed means and standard errors.

to plants having flowers with arrow-markings resulted in successful probings, but although some of the flies that approached plants having flowers without arrow-markings attempted to insert their proboscis into the perianth tube, only five out of 64 (7.8%) managed to do so successfully (figure 3*b*; $\chi^2 = 135.2, p < 0.0001$).

#### (ii) *Close-up choice tests*

The number of arrow-markings significantly affected which flower flies chose to probe (figure 3*c*; $\chi^2 = 41.23$, $p < 0.0001$) and all means differed significantly from each other (Dunn–Šidák test). There was an overwhelming predilection for 'intact' flowers with all six arrow-markings over flowers with zero arrow-markings, but flies also exhibited significant preference for intact flowers over flowers with only one arrow-marking and flowers with three arrow-markings (figure 3*c*).

### (**b**) ***Plant fitness***

#### (i) *Male fitness*

The probability of exporting dye grains depended greatly on whether arrow-markings were present or not. Only one out of 20 flowers without arrow-markings exported dye grains, to only one neighbouring flower, whereas 11 out of 21 flowers with arrow-markings exported dye grains (*G*-test: $G = 9.271$, $p = 0.0023$). Flowers with intact arrow-markings exported dye grains to an average of $1.8 \pm 0.98$ (range 1–4) neighbouring flowers.

#### (ii) *Female fitness*

The fruit set of flowers with arrow-markings (17 out of 29) was significantly higher than that of flowers without arrow-markings (nine out of 34; Fisher's exact test, two-tailed, $p = 0.0119$). In some of these fruits, one or two carpels had been attacked by seed predators, and so we assessed seed set only in fruits that had all three carpels intact. There was no significant difference in the number of seeds between fruits from flowers with or without arrow-markings (means and upper and lower standard errors obtained by back-transformation from the log scale: with arrow-markings: 9.106, +1.54, −1.86; without arrow-markings: 11.45, +1.47, −1.69; $\chi^2 = 1.06$, $p = 0.301$).

## 4. DISCUSSION

Our results clearly demonstrate the importance of the white arrow-markings on *L. oreogena* flowers for proboscis insertion by their pollinator. These arrow-markings could thus be considered to be functional nectar guides '*sensu stricto*'—i.e. visual markings on tepals of nectariferous flowers that guide flower visitors towards a concealed nectar reward. Interestingly, both tepals and arrow markings of *L. oreogena* are UV absorptive (figure 2) and thus contradict the popular notion that nectar guides are floral patterns that provide reflective contrast in the UV spectrum (cf. [4]). Moreover, our results provide clear evidence of a strong causal link between presence of nectar guides and both male and female components of plant fitness in a natural system.

For fly foraging, an absence of the nectar guides did not influence long-distance attraction (figure 3*a*). These results, attained in a natural system, are consistent with those from earlier studies that used artificial flowers [17,19,37]. However, once attracted, flies showed a clear-cut response to nectar guides: when guides were absent, flies hovered just above the corolla, sometimes attempting to probe the flower, but only very rarely being able to insert their proboscis in the perianth tube, thus reducing their foraging efficiency, while they invariably probed flowers when nectar guides were present (figure 3*b*). These results are even more pronounced than those of Manning [19] who found that, after initial attraction to a model flower, hovering bumble-bees 'dipped down' more often onto flowers with nectar guides than onto flowers without them. Despite this strong effect of nectar guides on fly behaviour, there were some fruits that developed in flowers of *L. oreogena* without arrow-markings. This is probably because their stigmas can be receptive for several days, and, despite being less likely to induce probing behaviour, these flowers still have a fairly high probability of being eventually pollinated when flies are as common as they were at our study site.

In the close-up choice tests, the response by the flies to the set-up of two adjacent flowers—one with all six nectar guides present versus a flower with none, one or three—varied with the number of nectar guides on the second flower (figure 3*c*). Whereas flies did not strongly discriminate between flowers with three or six nectar guides, they clearly preferred the flower with all six nectar guides versus flowers with none or one nectar guide. Interestingly, in the few cases when flies chose the flower with a single nectar guide, and successfully probed it, they often approached the set-up from the direction in which the tepal with the single nectar guide was pointed (D. Hansen 2007, 2008, personal observation). This suggests that flies are able to make their decisions rapidly during final approach, with only a single nectar guide clearly visible. It also raises the question whether a flower with three nectar guides would receive more successful visits if these nectar guides are located bilaterally on three adjacent tepals, or radially on alternating tepals. Future studies should address whether fly response depends on the symmetry of nectar guides.

Fitness of *L. oreogena* depended strongly on a presence of nectar guides, both for male and female components. Grains of the pollen-analogue dye were much more likely to be exported from donor flowers with nectar guides present than from donor flowers without them. However, we rarely found more than a few recipient flowers with dye grains within 50 cm around donor flowers. This pattern is probably explained by the foraging behaviour of the flies. They often fly/forage in almost straight lines across the landscape, and are therefore likely to visit only a few of the dozens of flowers that can occur within 1 m of the donor plant. The effect of nectar-guide removal on female fitness differed between fruit- and seed set. Whereas there was a large reduction in fruit set of flowers without nectar guides, there was no statistically significant decline in seed set per fruit. This is probably explained by the large amounts of *Lapeirousia* pollen grains carried by an individual fly (figure 1). A typical ovary has 20–25 ovules, and natural seed set per fruit is around 10–15 seeds. Therefore, it is likely that even a single visit by a pollen-carrying fly during a flower's lifetime would produce the observed seed set.

Very few studies have addressed the evolution of nectar guides. Our experimental approach simulates the appearance of mutants in the *L. oreogena* population. The results show that mutants with few or no nectar guides would be discriminated against by the specialist pollinator; a full complement of six nectar guides is thus under strong selective maintenance in this pollination system. This is probably further facilitated by the highly specialized pollination system with only one pollinator species, as this eliminates the possibility of pollinator-mediated disruptive selection on nectar guides (cf. *M. luteus*; [22]). In future studies, it would be interesting to simulate the appearance of mutants with nectar guides in a population of a suitable flowering plant species without visible floral nectar guides; for example, long-proboscid flies will probe flowers of uniformly coloured 'non-long-proboscid fly' plants (e.g. *Oxalis* spp., D. Hansen & T. Van der Niet 2007, 2008, personal observation).

One of the most striking convergent features of flowers pollinated by long-tongued flies is the presence of conspicuous nectar guides [28]. This suggests that visual cues may be particularly important in this pollination system, because these highly specialized flower visitors need something to guide their long, non-retractible proboscis towards the nectar reward. While very little is known about the perceptual dimensions of long-proboscid fly vision, it is clear that, given the length of its proboscis and common nectar guide widths of around 1–2 mm, *Prosoeca* sp. nov. must be able to detect objects with visual angles between 1.2° and 2.8°—comparing favourably, for example, with the capabilities of bumble-bees and butterflies [38,39].

It is not yet known whether the fly responses to nectar guides that were observed in this study are innate or learned. We think it plausible that long-proboscid flies have some innate responses to nectar guides as the adults are short-lived and depend entirely on nectar as a food resource. This would place a high selective premium on foraging efficiency and could explain why they form tightly specialized associations with particular plant species that flower precisely during the few weeks that the adult flies are on the wing [25]. Our results were obtained with experienced flies in a natural system. Establishing whether inexperienced flies have innate responses to nectar guides would require further behavioural studies with naive insects in a controlled environment. This is unfortunately difficult because the larval hosts of these flies are currently unknown, making it impossible to rear them in laboratories.

To conclude, our study firmly establishes a strong link between plant fitness and pollinator foraging behaviour mediated by visual nectar guides in flowers pollinated by long-proboscid flies. Experimental manipulation of visual nectar guides by changing their reflectance spectrum was employed in some earlier studies conducted in natural systems [9,21], but these focused solely on pollinator behaviour. Future studies of other systems that also incorporate the dimension of plant fitness will lead to an increased evolutionary understanding of why angiosperm flowers are so frequently characterized by visual floral guides.

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