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ORIGINAL PAPER

Neglecting nocturnal pollinators has long masked hawkmoth pollination in *Rhododendron*

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Abstract

Insufficient investigation of nocturnal floral visitors may bias our understanding of floral diversification in many plant lineages. Here we re-examined the pollination ecology of *Rhododendron excellens*, which lacks a narrow floral tube characteristic of many hawkmoth flowers and has been considered a bee specialist with daytime observations alone. We used five temporally sequential proxies (i.e. visitation rate, pollen deposition, fruit production, seed production, and seed viability) covering the entire process of reproduction to track the relative importance of the two floral visitor groups that were active by day and by night respectively. We then quantified the floral syndrome and tested the mating system with hand pollination treatments. Both bumblebees and hawkmoths regularly visited *R. excellens* in two flowering seasons. Hawkmoths' relative importance increased step-by-step from being inferior to bumblebees (floral visitation and pollen deposition) to making over three times the contribution by bumblebees (seed viability). This is probably because they differ in the ability to deliver outcross pollen for this partially self-compatible species. Correspondingly, *R. excellens* exhibits a floral syndrome associated with a particular subdivision of sphingophily. We revealed a distinctive pollination mode that mainly involves nocturnal pollinators and is likely to have repeatedly evolved in *Rhododendron*. Our findings also highlight the need to consider the 'quality' of pollen deposited onto stigmas, whose effect on plant fecundity may significantly expand over the course of sexual reproduction.

Keywords Nocturnal pollination · Pollination mode · Pollinator importance · *Rhododendron* · Sphingophily · Trumpet-shaped flower

Introduction

Many plant species have evolved floral traits for the attraction and exploitation of nocturnal pollinators. For instance, long tubular flowers with strong fragrance often attract hawkmoths (Van der Niet and Cozien 2022; Wang et al. 2023). We could therefore expect their interactions with those flower-visiting animals to take place in the dark (Willmer 2011). On the other hand, it has been increasingly realized that nocturnal floral visitors can be generalist foragers and actually visit many flowers with a syndrome clearly suggestive of some type of diurnal pollinators (Walton et al. 2020; Diniz et al. 2022; Souza et al. 2022). A number of studies (e.g. Jaca et al. 2020; Ohashi and Jürgens 2021) have demonstrated that diurnal pollination is more important but nocturnal visitors may play a supplemental role in the reproduction of plants (Scenario I). One would therefore miss a component of the pollination ecology of plants without fieldwork at night (Walton et al. 2020).

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Here we propose a second scenario in which nocturnal pollination is crucial to reproduction, with some floral traits indeed adapted to floral visitors foraging after sunset (Scenario II). However, floral visitation at night may also be overlooked due to the lack of a floral syndrome that can be exclusively associated with a certain type of well-known nocturnal pollinators (e.g. Liu and Huang 2013; Miller et al. 2014; Lombardi et al. 2017; Dellinger et al. 2019; Liu et al. 2019). In particular, if frequent visits by diurnal animals have already been found, one might not tend to pursue nocturnal floral visitation. The ignorance of what occurs in the dark will not only bias our understanding of floral ecology but also mask evolutionary events of flowers driven by nocturnal animals. For example, *Luffa acutangula* (Cucurbitaceae) does not conspicuously differ from its congeners in floral shape (saucer-shaped) and colouration (yellow) (Filipowicz et al. 2014) and has been considered to rely on bees to disperse pollen (Mitchell et al. 2015). However, its flowers open at dusk in contrast to all the congeners and were subsequently shown to be pollinated by a variety of moths as well as bees (Lu et al. 2021), representing a distinctive pollination mode in which night-flying insects are favoured.

In this study we re-examined the pollination ecology of *Rhododendron excellens* Hemsl. et Wils., which possesses large trumpet-shaped fragrant flowers. It has been observed being visited by large bees in the daytime, and therefore, like many congeners, was considered to rely on bees for pollination (Tian 2011). This floral syndrome also characterizes many other species in the genus and there have been no attempts to explore the interactions of such *Rhododendron* flowers with nocturnal animals in the literature. However, we are aware that hawkmoths pollinate plants that resemble these *Rhododendron* in the floral syndrome, such as some *Lilium* (Liu et al. 2019; 2022). In addition, our preliminary observations have found moth scales attached to stigmas of *R. excellens*, strongly indicating moth visitation.

We have two main objectives. (1) To reveal the relative importance of nocturnal and diurnal floral visitors in the reproduction of *R. excellens*. (2) To test for whether the floral syndrome is more related to bee or hawkmoth pollination. Then we can answer the question of whether the case of *R. excellens* is more consistent with Scenario I or II.

Materials and methods

Species and site

Rhododendron excellens (Ericaceae) is a shrub or treelet that occurs in the alpine forests or thickets in southwestern China and northern Vietnam, flowering in May. It has white fragrant trumpet-shaped corollas with anthers and stigmas positioned around the opening of the corolla. Each plant produces one or more flowering branches. Usually two to eight flowers cluster on the top of a branch that points upward, blooming largely at the same time. Flowers remain open for about a week. As in other *Rhododendron* species (Song et al. 2019), pollen grains are not free but attached to viscin threads. Anthers dehisce prior to anthesis so the viscin threads can be picked up as soon as the flower opens. The stigma, which presents sticky secretion and can readily catch pollen viscin threads once the flower opens, remains at the same position for the entire anthesis. Thus, the species shows no dichogamy but some extent of herkogamy.

Our study population is located in (23°09′32″N, 104°49′15″E; about 17 00 m a.s.l.), Ganbazi, Xiajinchang Town, Malipo County, Yunnan Province. Bee- and/or fly-pollinated Ericaceae, Theaceae, Rosaceae and Fabaceae with small non-fragrant flowers dominated the plants co-flowering with *R. excellens* in the community; *R. excellens* was the only blooming species that was attractive to hawkmoths and we never observed hawkmoths visiting other plants.

Flower visitation and pollen loads on hawkmoths

To compare visitation rates of diurnal and nocturnal visitors, we spent 22 and 18 h over four days in 2019 observing the former and the latter, respectively. Thirty-five flowers of one plant or 2–3 neighbouring plants when a single plant did not have enough flowers, were observed at intervals of one hour. We shifted to another patch of flowers after an interval had finished so each interval involved different plant individuals. In 2020, we spent 62 and 46 h over 12 days observing diurnal and nocturnal visitors. The observation time for nocturnal visitors was shorter because the period when we could work at night every day was comparatively limited and we sometimes encountered unfavorable weather. Observations of diurnal visitors were scattered between 09:00 and 19:15 h when they could be seen flying. Observations of nocturnal visitors were scattered between 19:30 and 23:30 h when they were active. Because visitation rates were relatively high this year, the number of observed flowers every hour was reduced to 20. We counted the number of visits to *R. excellens* flowers by diurnal and nocturnal insects within each hour of observation. To observe the visitors in the dark, we used a red-light LED flashlight (HuaenOpto, H-C15Li-R6, 5 w) covered with three layers of red plastic film. This setup did not disturb insects in our practice. We captured flower-visiting hawkmoths using a transparent plastic bottle with a wide mouth outside observation sessions. Then we looked at whether they carried pollen of *R. excellens* immediately in the field using tweezers and headlamps in 2020. We confirmed it if we found viscin threads on the moth.

Stigmatic pollen deposition

To compare the ability of diurnal and nocturnal floral visitors to deliver pollen for *R. excellens*, we bagged two branches using nylon net (apertures: 0.3 × 0.3 mm), each of which had four or more almost mature flower buds. We labeled four buds on each branch as the focal ones. One branch was bagged early morning and the other one late afternoon. Then the former was exposed to diurnal insects from 08:00 h next morning to 19:30 h on the same day, while the latter was exposed to nocturnal insects from 19:30 h the next day to 08:00 h. All the flower buds had already opened when we removed the bags to expose them to visitors. We repeated this procedure for 11 consecutive days so there were 11 plants in total for both diurnal and nocturnal exposure. All the flowering branches used in this experiment were from different individuals, i.e. 22 plants used altogether. We harvested these stigmas and fixed each of them in a 10 ml centrifuge tube with 0.5 ml of 70% ethanol. To detach deposited pollen from stigmas in the laboratory, we added 1 ml of 10% sodium hydroxide to the centrifuge tube containing a stigma and left the stigma to soak. We added 1 ml of 30% acetic acid to neutralize the sodium hydroxide for safety 20 min later. Then we shook the tube fiercely for a min to separate the pollen and the stigma. We took the stigma out and added water to the tube until the suspension increased to 4 ml. We took ten drops from this suspension with a dropper (0.48 ml in total) and counted the pollen grains they contained using glass slides and a microscope. Finally, with the number of the pollen grains (N) in the ten drops, we estimated the total of the pollen grains in the suspension of 4 ml (N × (4 ÷ 0.48)). There might be an amount of pollen left on the stigma so we treated the stigma further: we put it in another 10 ml centrifuge tube again and added 4 ml of water. Then we shook the tube as we had done at the beginning. We estimated the pollen grains in the same way as we described above. We repeated this procedure (usually two or three times) until we found no pollen grains in the suspension drops. We added all the estimates of pollen grains to calculate the estimated total of deposited pollen grains on the stigma.

Pollination treatments, fruit and seed production

To illustrate the structure of our data used in the next two parts, we present an example as below. If one treatment involves five flowers from one plant, and only three of them produced a fruit, we will record the fruit set as (1, 1, 1, 0, 0), with '1' and '0' indicating success or failure of fruit development respectively. If the three fruits contain 5000, 3000, and 1000 seeds, we will describe the seed production for the five flowers as (5000, 3000, 1000, 0, 0). If only half the seeds from the first two fruits and 30% of the seeds from the third fruit are viable, the numbers of viable seeds from the five flowers will be (2500, 1500, 300, 0, 0).

We conducted our pollination experiments in 2020. To compare the fruit and seed production due to pollination by diurnal and nocturnal visitors, we did two bagging treatments on each of 23 plants of *R. excellens*. We bagged one branch with flower buds in nylon net (apertures: 0.3 × 0.3 mm) from 19:30 to 8:00 h (the day exposure treatment) while the other from 8:00 h to 19:30 h (the night exposure treatment). This task lasted 7–9 days for each branch. We no longer bagged the branch once all of its flowers wilted. A third branch was not bagged and used as the control to determine whether there is a complementary effect of diurnal and nocturnal pollination. Here "a complementary effect" means that diurnal or nocturnal pollination alone cannot lead to the same level of fecundity as the control can bring about and their combination will therefore provide an advantage. We labeled three to six flowers on each branch for these treatments.

We harvested fruits in November 15 and calculated the fruit set. *Rhododendron excellens* has numerous dust-like undeveloped ovules, poorly developed seeds, and well developed seeds. We estimated seed production per fruit: we sieved the mixture of dry undeveloped ovules and seeds of each fruit was sieved in a mesh (apertures: 0.4 × 0.4 mm) to remove undeveloped ovules and some poorly developed seeds, which were smaller than developed ones. There was no clear morphological distinction between undeveloped ovules and poorly developed seeds, and between poorly developed seeds and well developed seeds and therefore this method was supposed to reduce subjective effects from researchers. We randomly took 100 seeds from this mixture of each fruit. We measured the weight of the 100 seeds and the weight of all the seeds, which were used to estimate the total number of seeds in the fruit. If the flower did not develop into a fruit, we recorded zero as the seed number. In addition, we made a supplemental experiment to determine whether some of those that could pass through the openings of the mesh were viable seeds (see Seed viability for the method). The results showed that they did not contain any seeds that could germinate and we did not mention this any more in Results.

To infer whether diurnal and nocturnal flower visitors differed in the ability to deliver outcross pollen, and to examine to what extent *R. excellens* is dependent on pollinators for reproduction, we also conducted three more treatments. Three branches in each of 11 plants were bagged. Three to six flowers in one branch were hand pollinated with pollen from three donors at least 20 m away and flowers (the cross pollination treatment), with pollen from the same plant (the self pollination treatment), or were prohibited from being visited by any insects using nylon net (the

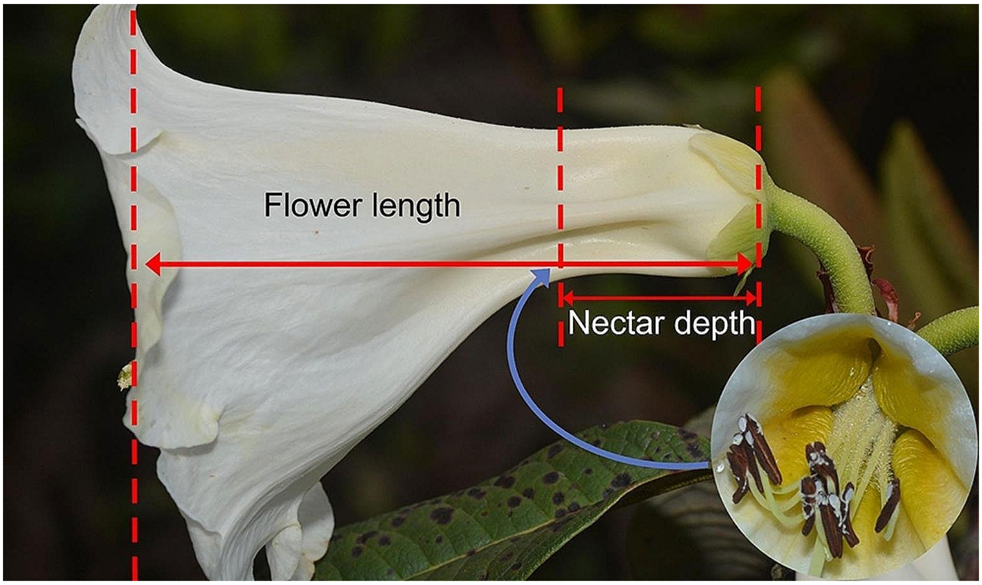


Fig. 1 How floral length and nectar depth were measured. The blue arrow shows where the corolla tube is sunken

pollinator-exclusion treatment). We investigated fruit set and seed production in the same way as mentioned in the previous paragraph.

Seed viability

To demonstrate how many viable seeds different pollination treatments produced, we evaluated seed germination using all fruits we obtained from the experiments aimed at testing diurnal vs. nocturnal pollination, and self vs. cross pollination. We randomly chose 200 seeds from each fruit. If a fruit had less than 200 seeds, all the seeds were used. The chosen seeds were dispersed on a plastic Petri dish of 90 mm diameter in the light (12 h light/12 h darkness, hereafter) under a fluctuating temperature regime (25/15°C). See Yang et al. (2020) for more details of the germination experiment. We estimated the number of viable seeds per fruit by the total seeds multiplying the percentage of seeds that could germinate. If the flower did not develop into a fruit, we recorded zero as the seedling number.

Table 1 Floral traits and the proboscis lengths of floral visitors. The percentage in the row of scent indicates the proportion of the scent blend that belongs to terpenoids

	Description	n	Mean ± SE
Flowers			
Floral length	Trumpet-shaped	40	84.6 ± 1.0 mm
Nectar depth	How deep the nectar is concealed	40	25.9 ± 0.5 mm
Scent	Dominated by terpenoids	12	74.04 ± 3.59%
Nectar volume	Standing crop at 19:30	40	32.7 ± 4.0 μL
Nectar concentration	Standing crop at 19:30	40	18.1 ± 0.5%
Floral visitors			
Bombus flavescens	Bumblebee	6	7.0 ± 0.1 mm
Acosmeryx naga	Hawkmoth	10	36.9 ± 0.3 mm
Cechetra lineosa	Hawkmoth	8	67.3 ± 1.0 mm
Cechetra scotti	Hawkmoth	7	68.9 ± 2.1 mm
Daphnis hypothous	Hawkmoth	7	51.2 ± 0.6 mm
Notonagemia analis	Hawkmoth	8	88.2 ± 2.4 mm

Traits of flowers and flower-visiting insects

To unravel which floral traits might be associated with favoring or discouraging diurnal/nocturnal visitors, we quantified floral morphology, nectar characters, and scent. (1) To examine the morphological fit between flowers and flower-visiting insects, we measured floral length of three flowers on each of 40 individuals with a plastic ruler and defined the length as the distance from the centre of the corolla opening to the base of the ovary (Fig. 1), with the average of the three flowers as a replicate. The corolla tube is sunken 2.5–3 cm from the base, forming narrow gaps within the corolla and apparently inhibiting large insects from crawling in. We therefore also measured the part from this point to the corolla base (nectar depth, see Fig. 1). Proboscises of 6–10 individuals of each of the flower-visiting insect species were uncoiled and measured (see Table 1 for the sample size of each species). Floral visitors were captured when visiting *R. excellens* or a UV light trap after all other field work. Some insect samples were from Liu et al. (2019). (2) To characterize the nectar volume and concentration, we measured the 19:30 nectar standing crop of one flower from 40 individuals using glass capillary tubes (1.0 mm in diameter) hand-held sucrose refractometer (0–50%, g solute per 100 g solution; Bellingham and Stanley Ltd., London, UK). (3) To detect the floral scent composition, we cut an inflorescence off each of 12 individuals in the afternoon and two newly open flowers were retained, with others removed. The branches were kept in a polyfoam box sealed up in adhesive tape with ice packs and carried to Kunming Institute of Botany, where floral scent was analyzed using solid-phase micro-extraction (SPME). The SPME holder with a 65 µm polydimethylsiloxane/divinylbenzene fiber (Supelco, Bellefonte, PA, USA) was inserted directly into a glass bottle with the two flowers from each plant to absorb the volatile compounds. Samples were analyzed using an Agilent Technologies HP 6890 gas chromatograph, equipped with an HP-5MS column (30 m×0.25 mm inner diam, 0.25 µm film thickness), and linked to an HP 5973 mass spectrometer. See Chen et al. (2015) for more details of the methods. All work on this experiment was finished in 24 h from the field collection of flowers.

Statistical analyses

We conducted the statistical analysis in R version 3.5.0 (R Core Team 2018). To compare floral visitor proboscis length with floral length and nectar depth, we used Mann-Whitney U tests (wilcox.test function in the 'stats' package). To compare visitation rates between diurnal and nocturnal floral visitors, we constructed a generalised linear model (GLM, glm function in the 'stats' package) with a Poisson distribution, and applied a logit link function. We analyzed data on stigmatic pollen deposition, seed numbers and seedling numbers in a generalised linear mixed effect model (GLMM) with a Poisson distribution and a logit link function. To compare fruits set of the diurnal and nocturnal exposure treatments and between fruit sets of the cross and self pollination treatments, we performed GLMM with a binomial distribution using the glmer function in the lme4 R package (Bates et al., 2015). During this analysis, we assigned plant ID and flower ID as a random effect, with flower ID nested within plant ID.

Results

Flower visitors and pollen loads on hawkmoths

Flowers of *R. excellens* were visited by bumblebees (*Bombus flavescens*) and honeybees (*Apis cerana*) by day and hawkmoths by night in 2019 (Fig. 2). No honeybees visited the flowers in 2020, but bumblebees and hawkmoths were still regular flower visitors. By vibrating the anthers, bumblebees collected pollen on their legs and abdomens (Fig. 2a). They contacted the stigmas at least on some occasions (Appendix S1; see Supplemental Data with this article). Notably, viscin threads with aggregated pollen sometimes hung from bumblebees (Appendix S2), suggesting that pollen can be deposited onto stigmas even without any contact between visitors and stigmas if these hanging viscin threads were captured by the sticky stigmas. In 2019, bumblebees often robbed the flower of nectar via the hole bitten on the base of the corolla (Fig. 2b) but nectar robbing was not found in 2020. Robbing visits were not included in the following comparison of visitation rate. Honeybees scavenged nectar flowing to the openings of the floral chamber (Fig. 2c). Occasionally they alighted on anthers (Fig. 2d), trying to collect pollen, but were never seen carry hanging viscin threads of pollen or contact the stigma. Thus, they did not transfer pollen onto stigmas and were not discussed later.

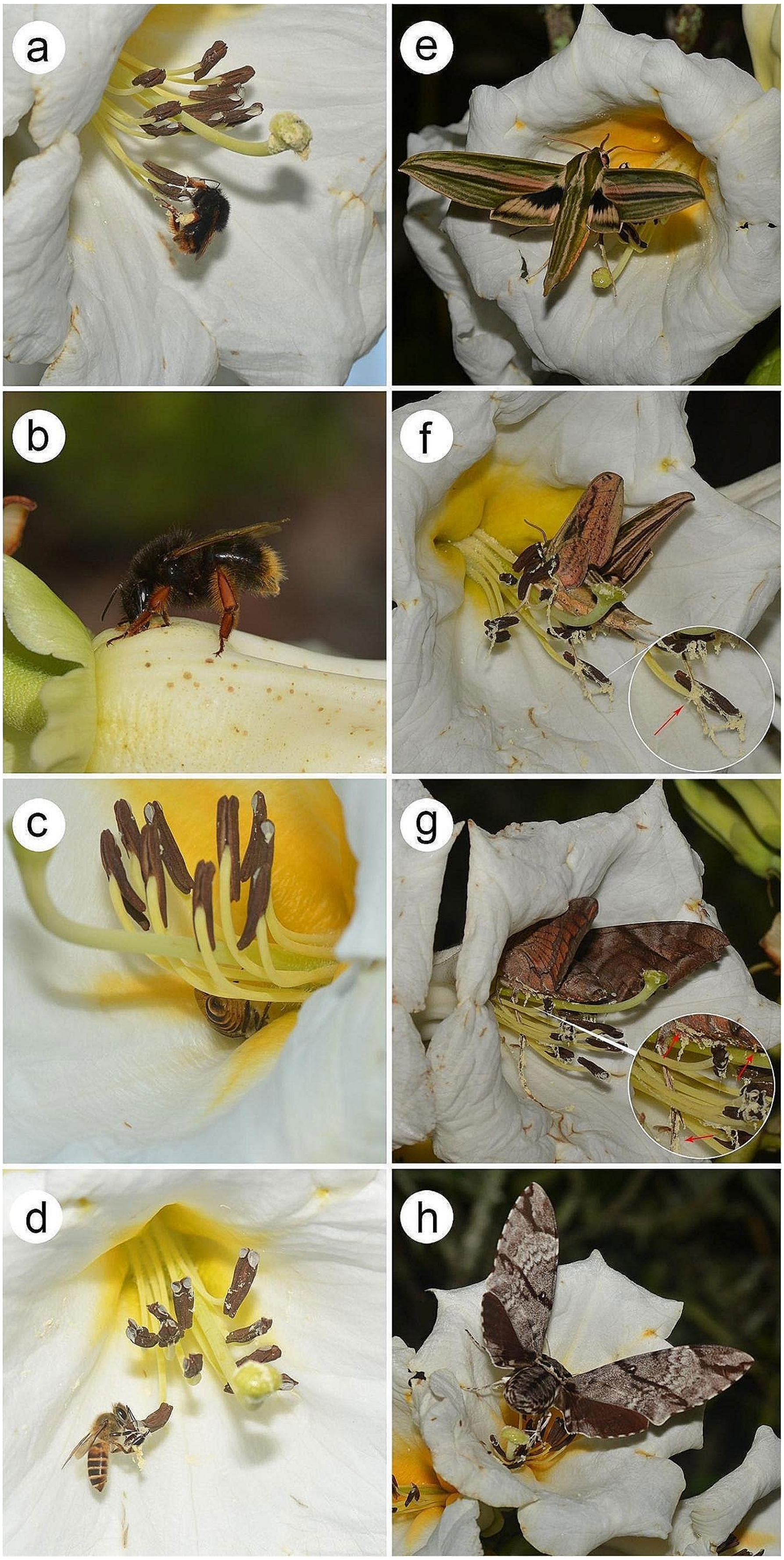
We recorded five species of hawkmoths as visitors altogether (Table 1). Hawkmoths usually entered the flowers and perched on the corolla, the style or the stamens, imbibing nectar through the openings of the nectar chamber (Fig. 2e, f, g). Hawkmoths also carried hanging viscin threads with aggregated pollen (Fig. 2f, g) as did bumblebee visitors. *Meganoton analis*, the hawkmoth with the longest proboscis, often hovered but still touched the anthers or stigma with the legs (Fig. 2h). The long proboscis of this species may prevent it from perching (Table 1). In total, we observed 21 hawkmoth individuals visiting flowers, belonging to the following species: *Notonagemia analis* ($n=6$), *Cechetra scotti* ($n=5$), *Acosmeryx naga* ($n=4$), *Cechetra lineosa* ($n=3$), and *Daphnis hypothous* ($n=3$). The check of pollen loads showed that every individual carried viscin threads with pollen except one *Cechetra lineosa* and one *Daphnis hypothous.* Viscin threads were attached to the legs, abdomens (Fig. 2f, g) or proboscises of the moths.

Comparison between bumblebees and hawkmoths

We observed floral visitors in 2019 and 2020 but other data were collected only in 2020. The observation results in 2019 were listed below in this paragraph and all data from the 2020 flowering season were presented in Fig. 3. Bumblebees ($n=62$ observation intervals) visited flowers more frequently than hawkmoths ($n=46$) in 2020 (GLM, Wald test, z = -5.651, $P<0.001$) (Fig. 3a). The difference was not significant in 2019 (GLM, Wald test, z=0.430, $P=0.667$) between bumblebees (mean ± SE: 5.5 ± 1.2 visits per hour, $n=22$) and hawkmoths (5.8 ± 0.9, $n=18$) Bumblebees deposited significantly more pollen grains per stigma than hawkmoths (both $n=44$ from 11 plants, GLMM, Wald test, z=25.51, $P<0.001$) (Fig. 3b).

In total, we used 105 and 108 flowers from 23 plants to test nocturnal and diurnal pollination, respectively. The night exposure treatment (i.e. hawkmoth pollination) did not differ significantly from the day exposure treatment (i.e. bumblebee pollination) in fruit set (GLMM, Tukey's test, z=4.633, $P<0.001$) and but did produce more seeds

Fig. 2 **a** *Bombus flavescens* collecting pollen; **b** *Bombus flavescens* robbing nectar; **c** *Apis cerana* scavenging nectar slipping to the entrance of the floral chamber; **d** *Apis cerana* collecting pollen; **e** *Cechetra scotti*, with legs contacting the anthers and stigma; **f** *Cechetra* sp. (*scotti* or *lineosa*), with viscin threads indicated by the red arrow; **g** *Acosmeryx naga*, with viscin threads indicated by the red arrow; **h** *Notonagemia analis*, with legs contacting the anthers



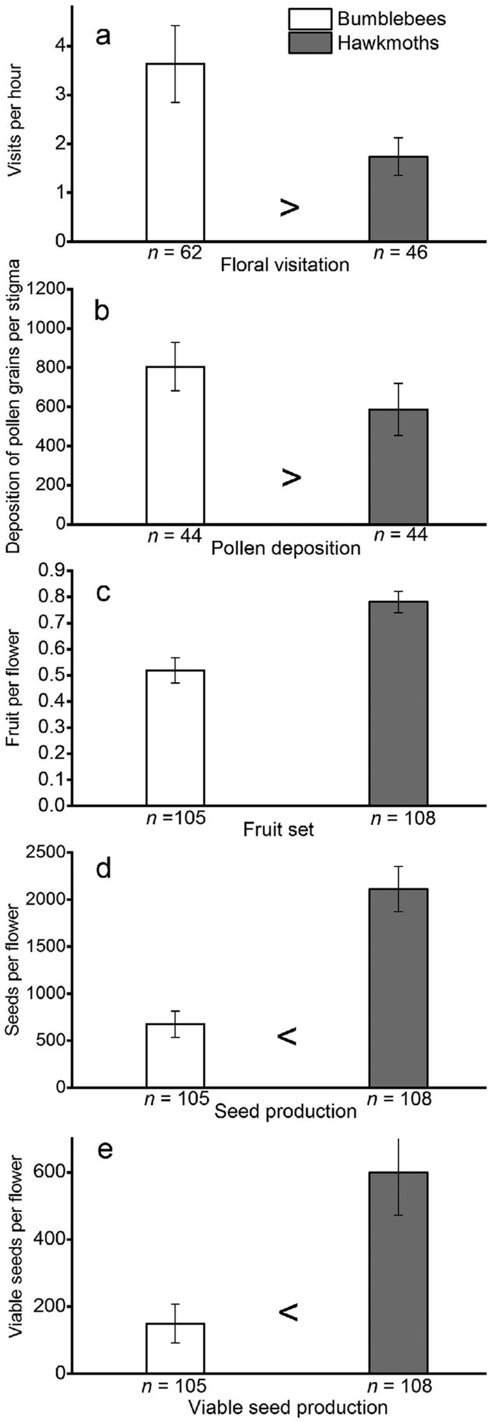


Fig. 3 The relative importance of bumblebees and hawkmoths as pollinators indicated by different proxies for pollinator importance in 2020. The data of visitation rate in 2019 were presented in the text (see Results). Columns with bars indicate mean ± SE. The numbers in the column refer to sample sizes, i.e. how many 1-hour observation intervals were made for visitation rates (**a**) and how many flowers are involved in those treatments (**b**, **c**, **d**). The flowers used to investigate pollen deposition were from 11 individuals. The flowers used to examine fruit set, seed production, and seed production were from 23 individuals. The "Visits per hour" means the number of visits to individual flowers per 1-hour interval within the observed patch containing 20 flowers. We use > and < to indicate "significantly more" and "significantly less". There is no significant difference if neither of them is used. The analysis of visitation rate in A did not include visits by nectar robbers

(z=3.101, $P=0.006$) and more viable seeds per flower (z=3.089, $P=0.006$) (Fig. 3c, d,e).

Test for complementary effects of diurnal and nocturnal pollination

To make Fig. 3 neat and straightforward to understand, we did not show the results of the control there but listed them as numbers below. The control (104 flowers from 23 plants) showed significantly higher fruit set (mean ± SE: 81.7 ± 3.8%) than the day exposure treatment (GLMM, Tukey's test, z = -5.049, $P<0.001$) but not than the night exposure treatment (z = -0.684, $P=0.773$). The control produced more seeds per flower (mean ± SE: 2485.7 ± 242.2) than the day (z = -3.102, $P=0.006$) and the night treatment (z = -3.095, $P=0.006$). The control brought about significantly more viable seeds per flower (mean ± SE: 606.8 ± 128.1) than the day (z = -3.089, $P=0.006$) but not than the night exposure treatment (z = -0.991, $P=0.582$).

Self-compatibility and autonomous self-pollination

The cross pollination ($n=49$ from 11 plants) and self pollination ($n=48$ from 11 plants) treatments did not differ significantly in fruit set (GLMM, Wald test, z=0.535, $P=0.593$) but the former resulted in higher seed production (z = -330.060, $P<0.001$) and more viable seeds per flower than the self pollination treatment (z = -174.514, $P<0.001$) (Fig. 4). No fruits were produced following the pollinator-exclusion treatment, suggesting that *R. excellens* is not capable of autonomous self-pollination.

Floral traits and proboscis lengths of floral visitors

Rhododendron excellens secreted relatively dilute abundant nectar (Table 1). A total of 71 volatile compounds were detected in its floral scent, among which 1,8-Cineole, Sabinene, and α-Terpineol were the three most abundant (Appendix S3). Terpenoids accounted for nearly three-quarters the total blend (Table 1, Appendix S3).

Bumblebees ($n=6$) had tongues that were much shorter than the nectar depth ($n=40$, z=278 -3.914, $P<0.001$) (Table 1) while the proboscises of all the hawkmoth species were significantly longer than the nectar depth (*Acosmeryx naga* z = -4.839, $n=10$; *Cechetra lineosa* z = -4.413, $n=8$; *Cechetra scotti* z = -4.169, $n=7$; *Daphnis hypothous* z = -4.169, $n=7$; *Notonagemia analis* z=4.413, $n=8$; all

$P < 0.001$) (Table 1). Floral length ($n = 40$) was significantly longer than the proboscises of hawkmoth species (*Acosmeryx naga* z = -4.852, *Cechetra lineosa* z = -4.372, *Cechetra scotti* z = -4.035, *Daphnis hypothous* z = -4.184, all $P < 0.001$) except for *Notonagemia analis* (z = -1.439, $P = 0.150$).

Disscussion

Rhododendron excellens attracted not only bumblebees but also hawkmoths as flower visitors that had chance of contacting stigmas and effecting pollen deposition in both flowering seasons. In 2020, bumblebees showed higher visitation rate and delivered more pollen onto stigmas, but hawkmoths were by far the more important pollinators if seed production and especially seed viability were considered. A rather low level of self-compatibility was detected. Quantification of floral traits showed that *R. excellens* exhibits a syndrome largely favoring hawkmoth pollination.

Floral visitors

Bumblebees have generally been documented as floral visitors to *Rhododendron* species and therefore it is not surprising that they were also common diurnal visitors to *R. excellens* (Escaravage et al. 1997; Ng and Corlett 2000; Stout 2007). They usually acted as the important pollinators of these species even in species with the bird pollination syndrome (e.g. Huang et al. 2017), indicating that bumblebee pollination might be the predominant pollination mode in *Rhododendron*. Honeybees were also floral visitors to several other *Rhododendron* but unlike what we saw in *R. excellens*, it is unknown whether honeybees were able to disperse pollen for those species.

A North American and a European *Rhododendron* species have been reported to be visited by nocturnal hawkmoths and settling moths, respectively (Grant 1983; Mejías et al. 2002). However, the relative frequency of diurnal and nocturnal visitors was not quantified. Our study is the first to document flower-visiting behaviours at night for the genus in Asia and we found that night-flying hawkmoths were main floral visitors to *R. excellens* (Fig. 3), together with bumblebees. All the five hawkmoth species are common and widespread in southeastern Asia (Pittaway and Kitching 2018). The only previous study that has mentioned visits by hawkmoths to Asian *Rhododendron* involves just several day-flying hawkmoths (Ng and Corlett 2000). We could expect that night-flying hawkmoths are likely to visit many other Asian *Rhododendron* species because hawkmoths are generalist nectar foragers, and there is apparent overlap between the flowering seasons of these plants and the seasons when hawkmoth adults are active (see Fang et al. 2005; Pittaway and Kitching 2018).

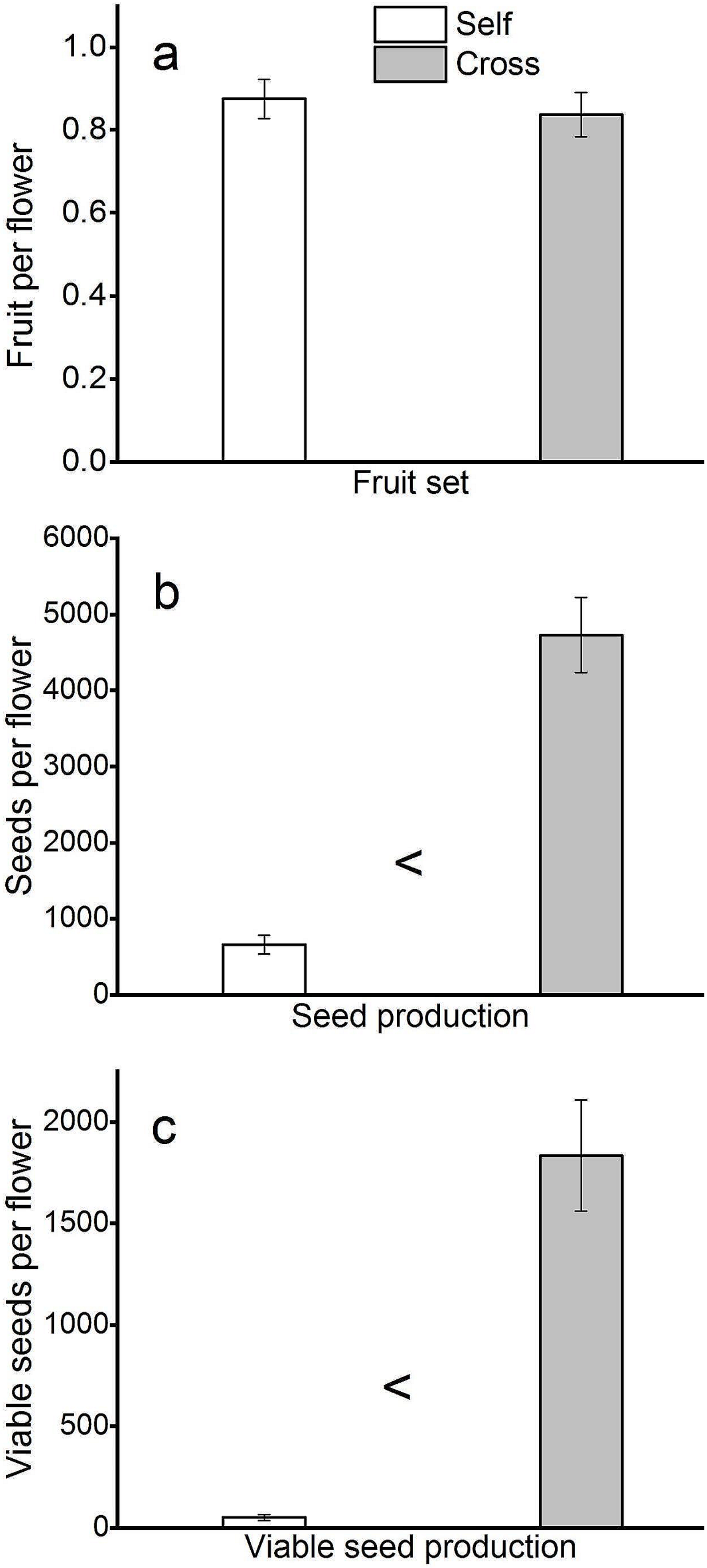
Nocturnal vs. diurnal pollination

It is common that flowers are visited by both diurnal and nocturnal animals, whose relative importance to plant fecundity is highly variable, depending on plant species (reviewed in Young 2002; Liu and Huang 2013; Jaca et al. 2020; Ohashi and Jürgens 2021). According to the comparison based on visitation rate and pollen deposition, bumblebees were more important than or similarly important to hawkmoths (Fig. 3a, b). However, hawkmoths mattered more when fruit and seed productions are considered. If seed viability is included, the relative importance of hawkmoths rises further, exceeding three times that of bumblebees, apparently supporting the predicted association between the floral syndrome and the pollinator identity. According to the proposal by Fenster et al. (2004) and Reynolds et al. (2009), one can define a plant species as a pollination specialist if one pollinator group such as bees or birds alone accounted for more than 75% of pollination of this species. This is obviously the case in *R. excellens* (Fig. 3). Overall, our findings highlight the need to consider the 'quality' of pollen deposited onto stigmas, whose effect on plant fecundity may significantly expand over the course of sexual reproduction.

Self-compatibility and its implications for pollination

Self-pollination resulted in substantial fruit and seed production in *R. excellens*, which is similar to the findings in other *Rhododendron* plants (Escaravage et al. 1997; Ng and Corlett 2000; Stout 2007). On the other hand, despite fairly high levels of fruit and seed production (Fig. 4a, b), viable seeds per flower from self-pollination turned out to be equal to just around 3.1% of those from cross-pollination per flower (Fig. 4c). Thus, the sequential reduction of bumblebee's relative importance from 'pollen deposition' to 'seed viability' (Fig. 3b), strongly suggests that bumblebees deposited more proportions of selfing pollen than hawkmoths. In fact hawkmoths have long been known as good dispersers of outcross pollen (Brunet and Sweet 2006; Skogen et al. 2016). Seeds from selfing and outcrossing may display differential viability (Dudash 1990; Culley et al. 1999); moreover, there is often difference in the ability to effect outcross pollination between pollinator groups (Brunet and Sweet 2006; Diller et al. 2022). Our study therefore adds to evidence that the ratio of outcross to self pollination can be a key component in evaluating a pollinator's role in enhancing plant fitness (Matsuki et al. 2008; Diller et al. 2022).

Fig. 4 The comparison between cross and self pollination with respect to three proxies, i.e. fruit set (**a**), seed production (**b**), and viable seed prodution (**c**). Columns with bars indicate mean ± SE. This experiment was conducted using 44 flowers from 11 plants, each of which were treated for both cross and self pollination. We use < to indicate "significantly less"



Are nocturnal and diurnal pollination complementary to each other?

For some plants that are visited in both the daytime and nighttime, diurnal or nocturnal pollination alone cannot result in as much fecundity as natural pollination (e.g. Ortiz et al. 2000; de Avila and Freitas 2011; Ohashi and Jürgens 2021). Plants therefore explicitly benefit from such a diurnal-and-nocturnal pollination mode. Nevertheless, this is not the case for *R. excellens*, in which pollination by bumblebees not only resulted in lower levels of fecundity than the control, but also failed to bring about an advantage for the control over hawkmoth pollination overall (see Test for complementary effects of diurnal and nocturnal pollination). Similar findings have been reported in a few other plants (e.g. Jaca et al. 2020; Funamoto and Sugiura 2021) and these authors argued that nocturnal and diurnal insects have a complementary effect on pollination. Given that pollinator populations often fluctuate greatly between years, significant complementary effects may be detected in some years in these plants, including our focal species here.

Although the control performed better than the pollination by hawkmoths in seed production, the test for seed viability revealed that this did not translate into a bigger total of seeds per flower that were able to germinate in the control (see Test for complementary effects of diurnal and nocturnal pollination). Again we could expect that bumblebees delivered more selfing pollen grains onto stigmas. Selfing pollen grains may have clogged some stigmas and/or fertilized some ovules (LLoyd 1986), causing a detrimental effect on pollination by hawkmoths.

Moreover, as pollen collectors, bumblebees intentionally transfer considerable amounts of pollen to their corbicula (i.e. pollen baskets) (Fig. 2a), which are no more available for pollination (Lopezaraiza–Mikel et al. 2007). In fact it has been corroborated that pollen-collecting bees themselves can be the source of pollen limitation since they can heavily reduce the amount of pollen that can be transferred by more efficient pollinators (Koski et al. 2018). Thus, the bumblebee is likely not to contribute positively to the fitness of *R. excellens* in the presence of abundant hawkmoth pollinators. This needs to be explored with more detailed experimental designs in the future.

Hawkmoth pollination and its putative evolution in *Rhododendron*

Given that pale coloration and fragrances can also occur in plants pollinated by large bees (Vogel 1954; Hendel-Rahmanim et al. 2007; Chain-Guadarrama et al. 2019) and *R. excellens* lacks narrow tubular flowers characteristic of common sphingophilous flowers (e.g. Skogen et al. 2016; Van der Niet and Cozien 2022; Wang et al. 2023), it seems somewhat reasonable for Tian (2011) to assign *R. excellens* to the bee-pollination syndrome. However, as shown by this study, it secretes plenty of dilute nectar accessible only to long-tongued visitors, emitting a sweet floral scent dominated by terpenoids (especially oxygenated) (Table 1; Appendix S3); several relatively abundant terpenoids, including 1,8-Cineole, Sabinene, α-Terpineol, and trans-β-Farnesene are also characteristic of some other sphingophilous flowers (Dudareva and Pichersky 2006). The sunken points in the corolla act as a morphological filter against nectar feeders except for hawkmoths despite the lack of a long narrow tube (Fig. 1). Thus, these traits constitute a syndrome in favour of hawkmoth visitation. In fact *R. excellens* can readily be categorized into a particular type of sphingophilous plants (reviewed in Liu et al. 2019) with large trumpet-shaped flowers pollinated by hawkmoths with proboscises of various lengths (Fig. 2; Table 1). In addition, pollen is attached to various parts of the moth body (Fig. 2) and all the hawkmoth species as floral visitors can carry pollen as shown by our checking out the captured hawkmoths (see Flower visitors and pollen loads on hawkmoths), which has also confirmed in other plants with huge trumpet-shaped flowers (e.g. Eggli and Giorgetta 2015; Liu et al. 2019). Such a large pale broad corolla tube (approx. > 4 cm long) occurs in many other species in different *Rhododendron* clades in mainland Asia (Fang et al. 2005), implying floral convergence as a result of adaptation to hawkmoths, but further fieldwork is required to test for it.

In addition, in Sect. Vireya of *Rhododendron*, which mainly occurs in the Malay Archipelago, some species with pale salver-form flowers have been hypothesized to be pollinated by hawkmoths (Stevens 1976). However, they constitute a subdivision of sphingophily that is morphologically different from *R. excellens*, and their pollination ecology has never been studied in the field. If hawkmoths are confirmed as pollinators in these species in the future, fascinating questions will emerge: e.g. How do these two guilds of sphingophilous *Rhododendron* differ in interacting with hawkmoths? Why have these two guilds of sphingophilous *Rhododendron* evolved in different clades of the genus and different geographical zones?

Concluding remarks

We revealed what had been missed by Tian (2011) without field work at night: *R. excellens* attracted hawkmoths as well as large bees as regular flower visitors, and hawkmoths were the principal pollinators. Our findings demonstrate how the pollination mode of a plant ‘changed’ from bee, to bee-hawkmoth bimodal, and finally to hawkmoth pollination as more details of pollination ecology were considered.

Rhododendron excellens displays high level of floral specialization for hawkmoths and is likely representative of a guild of species sharing the same pollination mode in *Rhododendron*, which had been neglected by previous studies in the genus. In summary, our findings are consistent with Scenario II: Nocturnal pollinators are key to plant reproduction and have driven floral evolution to some extent, but flowers show no syndrome that fits classic description of hawkmoth pollination and the plant-pollinator interaction in the dark may therefore tend to be neglected.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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