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The lady's 'slippery' orchid: functions of the floral trap and aphid mimicry in a hoverfly-pollinated *Phragmipedium* species in Brazil

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• **Background and Aims:** Trap flowers are fascinating cases of adaptation, often linked to oviposition-site mimicry systems. Some trap flowers do not imprison pollinators for a pre-determined period, but rather force them to move through a specific path, manipulating their movements in a way that culminates in pollen transfer, often as they leave through a secondary opening.
• **Methods:** We investigated the previously unknown pollination system of the lady's slipper orchid *Phragmipedium vittatum* and assessed the function of micro-morphological traits of its trap flowers.
• **Key Results:** Our observations revealed that *P. vittatum* is pollinated by females of two hoverfly species (Syrphidae). Eggs laid by flies on or near raised black spots on the flowers indicate that the orchid mimics aphids which serve as food for their aphidophagous larvae. Dark, elevated aphid-like spots appear to attract the attention of hoverflies to a slipping zone. This region has downward projecting papillate cells and mucilage secretion that promote slipperiness, causing potential pollinators to fall into the labellum. They then follow a specific upward route towards inner aphid-like spots by holding onto upward oriented hairs that aid their grip. As hoverflies are funnelled by the lateral constriction of the labellum, they pass the stigma, depositing pollen they may be carrying. Later, they squeeze under one of the articulated anthers which places pollen smears onto their upper thorax. Then, they depart through one of the narrow lateral holes by holding onto hairs projecting from the petals.
• **Conclusions:** This study confirms the system of aphid mimicry in *Phragmipedium* and highlights the sophisticated micro-morphological traits used by trap flowers in pollinator attraction, trapping, guidance and release, thus promoting precise pollen transfer.

Key words: Cypripedioideae, trap flowers, floral mimicry, floral traits, Cypripedioideae, Syrphidae, trap flowers.

INTRODUCTION

> "*The labellum thus acts like one of those conical traps with the edges turned inwards*"
> Darwin (1862) when referring to a lady's slipper orchid

Floral traps are among the most sophisticated mechanisms for enabling effective pollination and are closely, but not exclusively, associated with various strategies of floral deception (Bröderbauer *et al.*, 2012). The key innovation of trap flowers is the chamber wherein pollinators enter actively or passively, remaining for a shorter or longer time, and where they contact the reproductive parts hidden inside (Faegri and Van der Pijl, 1979; Endress, 1996; Bröderbauer *et al.*, 2012; Johnson and Schiestl, 2016). Trap flowers evolved multiple times, being distributed in at least 11 plant families (Faegri and Van der Pijl, 1979; Endress, 1996; Richards, 1997; Bröderbauer *et al.*, 2012; Johnson and Schiestl, 2016).

Flowers with 'perfect traps' imprison pollinators for longer pre-determined periods and then allow them to escape when structures change after the plant switches the sex phases (Vogel, 1965; Faegri and Van der Pijl, 1979). By contrast, flowers with 'semi-traps' (imperfect traps) do not imprison pollinators, but rather force them to take a specific route through a floral labyrinth within the trap (Faegri and Van der Pijl, 1979). This culminates in pollen deposition and then removal as pollinators manage to leave the flower, usually using an exit formed by another floral aperture. These flowers typically have narrow passages that restrict pollinator size, direction and positioning, allowing precise and accurate pollen transfer.

The pollination cycle of a semi-trap flower can be divided into three pre-determined phases, each of them involving specific morphophysiological traits (Fig. 1). First, the pollinator must be attracted to, enter and be trapped in the flower. The second phase consists in guiding the pollinator along a specific path within the floral chamber, leading to precise and accurate pollen transfer in tight regions where the pollinator must squeeze through. Finally, the third phase consists of the pollinator leaving the flower, where it must keep following the set route until it arrives at the exit hole.

Semi-trap flowers employing the three-phase strategy are common in some families such as the Orchidaceae where they

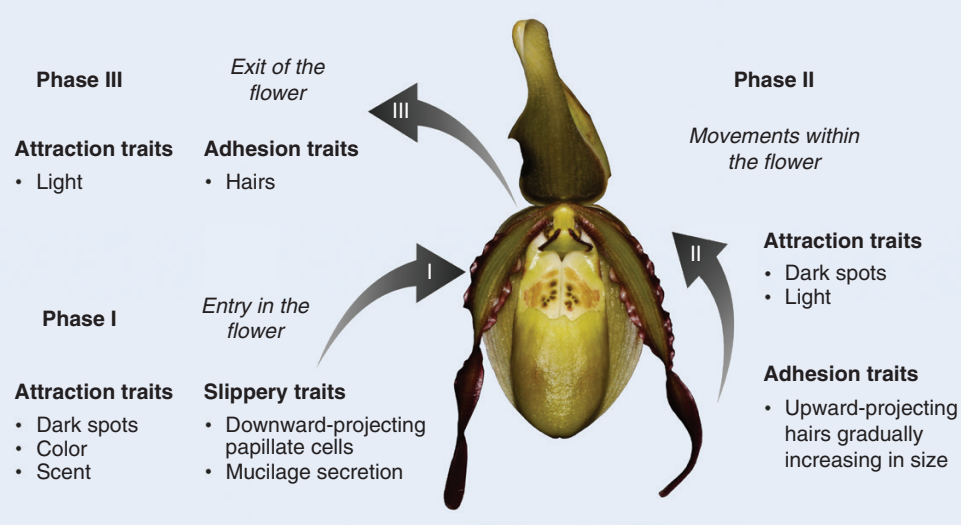

FIG. 1. Phases of pollination of a semi-trap flower, using *Phragmipedium vittatum* as an example to highlight traits that mediate pollination.

are present in many unrelated lineages (Faegri and Van der Pijl, 1979). Many of these species employ brood-site mimicry, such as the case of *Gastrodia similis* which mimics the scent of rotting fruit in order to lure drosophilid fly pollinators into a touch-sensitive floral trap (Martos *et al.*, 2015). Some studies report sexual mimicry associated with orchid trap flowers. For instance, *Pterostylis* spp. (Orchidoideae) attract males of fungus gnats that try to copulate with the labella, triggering its closure and trapping insects inside the flower (Phillips *et al.*, 2014; Reiter *et al.*, 2019). In another case, the upward-oriented flowers of *Trigonidium obtusum* (Epidendroideae) attract males of a eusocial stingless bee that slip on the perianth surface and become trapped in the flower cavity (Singer, 2002). Other attractants may involve fragrances, such as the case of *Coryanthes* (Epidendroideae), which trap Euglossini bees that fall into the fluid-filled labellum when trying to collect such resources (Gerlach, 2011).

Trap flowers are widespread in the orchid subfamily Cypripedioideae, where the modified pouch-like labellum is a synapomorphic trait (Dressler, 1981; Fig. 1). The labellum usually plays a fundamental role in trapping pollinators and assuring cross-pollination of the rewardless flowers in most species via food or brood-site mimicry (Pemberton, 2013; Edens-Meier *et al.*, 2014). In the Cypripedioideae, pollination systems are relatively well known for some temperate and subtropical *Cypripedium* and *Paphiopedilum* species (Bänziger *et al.*, 2012; Pemberton, 2013; Edens-Meier *et al.*, 2014; Jiang *et al.*, 2020; Zheng *et al.*, 2022). However, knowledge about pollination in the neotropical genus *Phragmipedium* is scarce (Pemberton, 2013), particularly with respect to the modes of pollinator attraction and the pollination mechanisms, including which micro-morphological mechanisms cause slipperiness and guide pollinators through the trap system. So far, it is known that some species are autogamous while others are pollinated by hoverflies or a mixture of hoverflies and bees (Pemberton, 2011, 2013). Due to the spotting on the infolded lobes of the labellum of some species, aphid mimicry was suggested as the mechanism that deceives female hoverflies, which search for oviposition sites to supply their aphidophagous larvae (Pemberton, 2011, 2013; Morales, 2017), although this has not been investigated in depth. *Phragmipedium* is the third most speciose genus of slipper orchids (Pemberton, 2013), and information on its pollination systems is important for better understanding the evolution of floral traits in Orchidaceae.

As a first step towards filling these knowledge gaps, we investigated the reproductive biology and complex pollination mechanism of the rare and vulnerable South American species *Phragmipedium vittatum*. We used this species to address the following questions: What are the pollinators and are they attracted by floral mimicry? What is the breeding system and is the plant dependent on pollinators for seed production? How does the trap mechanism function and what are the roles of micro-morphological perianth features?

MATERIALS AND METHODS

Study species and site

Phragmipedium vittatum (Vell.) Rolfe (Cypripedioideae) is a paludal herb that occurs mainly in Veredas (occasionally in forests) vegetation of the Brazilian Cerrado biome, characterized by a dense herb–subshrub dominant layer, with scattered small trees (Supplementary Data Fig. S1) (Araújo *et al.*, 2002). It is a rare lady's slipper species, considered vulnerable to extinction (Dias and Smidt, 2020). *Phragmipedium vittatum* flowers once a year, between October and May, but peaking in December–February. Flowers open acropetally, with a mean (± s.d.) number of 6.86 ± 2.22 flowers per individual and a mean inflorescence height of 74.79 ± 16.87 cm (n = 100). Pre-bagged flowers lasted an average of 9.46 ± 0.86 days (n = 26). After approximately 6 d, flowers senesce and their original colours fade, detaching from the stem a few days later. After detachment of a flower, another opens, and usually a single flower is opened at a time per inflorescence. Plant individual flowering time depends on the number of flowers on the inflorescence, lasting ~1.5–2 months. Average flower size, measured as the longitudinal size

of the labellum, was 3.87 ± 1.69 cm (n = 507) (see Table S1 for additional flower measurements). Labellum colour ranges from greenish, through yellowish to brownish, with wavy lateral petals bordered by dark reddish brown at the base and fully coloured at the tip.

We studied a population located on a private farm in Goiás state, Brazil, during December 2017 – January 2021. The region has a seasonal climate (AW in the Köppen-Geiger classification), with rainy summers and dry winters (Alvares *et al.*, 2013).

Pollinator observations

During 2018 and 2019, we performed 56 observation hours between 0800–1200 h and 1400–1800 h (totalling 7 d). Observations were conducted by the same person, at ~1.5 m from a given patch containing approximately five open flowers. We recorded the frequency and behaviour of pollinators and noted if they were carrying pollen smears. Due to the low frequency of pollinator visits found through the direct observation method, in 2021 we conducted an experiment to assess flower visitation rates on a larger number of flowers. For 2 d, we stuffed cotton wool into both exit holes of previously bagged plants, which prevented any trapped pollinator from escaping via the exit holes. We unbagged flowers at 0600 h and recorded the presence and identity of pollinators every 3 h until 1800 h. This was done for 47 flowers on the first day and 42 flowers on the second day. We also recorded the presence of eggs laid by pollinators on the flowers, as this would be an indication of a brood-site deception system.

Pollinator effectiveness

To estimate the effectiveness of pollinators for male and female components of pollination success, we inspected flowers during the 2016–2017 (n = 306 individual flowers), 2017–2018 (n = 161), 2018–2019 (n = 301) and 2020–2021 (n = 67) flowering seasons. We selected flowers at the end of their lifespan. Pollen removal (male success) was estimated from pollen removal from either one or both anthers. Pollen deposition (female success) was estimated from the presence of pollen smears on the stigma of senescent flowers.

Breeding system and pollen limitation

The breeding system was investigated in December 2020 by submitting newly opened flowers of previously bagged buds from different individuals to one the following hand-pollination treatments (*sensu* Kearns and Inouye, 1993): (1) cross-pollination: the pollen smears of one anther from another plant located at least 20 m away were inserted in the stigma (n = 17); (2) self-pollination: the pollen smears of one anther were inserted into the stigma of the same flower (n = 13); (3) spontaneous self-pollination: buds were only bagged, without any manipulation (n = 21); and (4) emasculation: the pollen smears of both anthers were removed, without subsequent treatment (n = 17). We use 'pollen smears' instead of pollinia throughout the paper because, technically, there is no pollinarium in the Cypripedioideae as pollen is loosely held together and the pollen mass is not attached to a viscidium (Dressler, 1981). We also investigated the reproductive success of flowers from open-pollination by inspecting fruit formation on 72 flowers from 22 individuals. The self-incompatibility index (ISI) was determined from the ratio of the percentages of self- and cross-pollinated flowers that developed fruits subtracted from 1, with values above 0.8 indicating self-compatibility (*sensu* Lloyd 1965). The pollen limitation index (PL) was calculated by dividing the percentages of open-pollination fruit set by those of manual cross-pollination subtracted from 1, with values above 0.8 indicating pollen limitation (*sensu* Larson and Barrett, 2000).

We took ripe fruits from the different treatments (eight self-pollinated, ten cross-pollinated and nine open-pollinated) just before the capsule opening process (April 2017). Fruits were placed in separate vials until they fully opened. We estimated seed viability by submitting 500 fresh seeds per fruit to 1.0 % (w/v) aqueous solution of 2,3,5-triphenyltetrazolium chloride for 24 h (*sensu* Lakon, 1949). Seeds with stained embryos were considered viable while those with unstained embryos or without embryos (i.e. empty seeds) were considered unviable. All the seeds of a fruit were separated (including those used in viability analysis and those remaining in the capsule), then immersed in a 50-mL solution containing foure parts ethanol 70 % and one part glycerine. This solution provides a homogeneous distribution of the seeds after mixing. Three aliquots of 0.25 mL were taken per fruit using a volumetric pipette, the number of seeds with well-developed coats was counted using a microscope and the mean value was then calculated. The proportion of the total volume was then calculated to estimate the total number of seeds per fruit.

Histological and histochemical analysis

We visually inspected opened flowers searching for any floral reward. We investigated the presence of secretory tissues by immersing fresh flowers in 0.1 % (w/v) aqueous neutral red for 20 min (Vogel, 1962; time adapted). To detect mucilage location, we immersed the fresh flowers in ruthenium red for 5 min (Gregory and Baas, 1989; time adapted). We submitted longitudinal handmade cuts from flower samples to histochemical tests to detect chemical compounds related to pollination process. To investigate the presence of nectar, tests were carried out for reducing sugars (glucose, fructose) with Fehling reagent (Purvis *et al.*, 1964), and starch grains with Lugol (Johansen, 1940). We used Periodic acid-Schiff's (PAS) to test for the presence of total insoluble carbohydrates (Johansen, 1940), Sudan red for total lipids (Sass, 1951), Bromophenol blue for total proteins (Johansen, 1940) and NADI reagent for terpenoids (David and Carde, 1964). For all histochemical tests, appropriate controls were run simultaneously. Light microscopy observations were carried out using a coupled Uphoto system (Leica ICC50HD).

For histological studies we made free-hand sections of sepals, labella and other petals, clarified in sodium hypochlorite (Johansen, 1940), and stained them with Safranin and Astra blue (Gerlach, 1984). Flowers were also examined using scanning electron microscopy (SEM). The alcohol-fixed parts were dehydrated in an ethanol series, critical point-dried (using a Leica CPD 300) and then mounted on aluminium stubs with conductive adhesive. Samples were coated with gold in a

sputter coater (Leica EM SCD050) and photographed using a scanning electron microscope (Zeiss EVO MA10) at 5 kV.

Statistical analysis

To determine the breeding system of *P. vittatum*, we compared the proportions of fruit set among those treatments that successfully set at least one fruit (spontaneous self-pollination and emasculation excluded) through a GLM (generalized linear model) with binomial error distribution and logit link function. As the response variable, we took the number of flowers that developed into fruits relative to the number of flowers that did not per individual using the *cbind* function in the R software base package (Crawley, 2013). We used this same procedure to investigate differences related to seed viability according to treatments that formed fruits: we took the number of viable seeds relative to the number of unviable per fruit (i.e. the proportion of viability) as the dependent variable and fitted a quasibinomial GLM (to account for overdispersion) with logit link. Differences in the number of seeds among treatment levels were investigated by fitting a GLM with negative binomial distribution (linear parameterization) and log link using the R-package glmmTMB v.1.0.2.1 (Brooks *et al.*, 2017). We accessed the significance of models using type II tests in the R-package *car* v.3.0.10 (Fox and Weisberg, 2019) and conducted *post hoc* analyses using the Tukey multiple comparison test in the R-package *multcomp* v.1.4.13 (Hothorn *et al.*, 2008). All analyses were carried out in R v.4.0.2 (R Core Team, 2020).

RESULTS

Pollinators

During our systematic pollinator observations, we found that *P. vittatum* was pollinated by females of two hoverfly species (Diptera: Syrphidae): *Allograpta exotica* (Fig. 2A–J) and *Dioprosopa clavata* (Fig. 2K–N). In our direct observations, we recorded 20 approaches from *A. exotica*. They hovered in front of the flower seven times (35 %) and landed in one of the whorls 13 times (65 %), never falling into the pouch. Three out of those 20 flies (15 %) had pollen smears attached to the upper part of their thorax. Photos and videos of *A. exotica* were taken during random encounters that occurred in periods other than those 56 h utilized for systematic observations. For *D. clavata*, we recorded three approaches. In one of them, the fly only hovered while in the other two they did fall and were trapped within the pouch. None of them had pollen smears attached.

In our exit hole blocking experiment, we found a total of 22 individuals trapped. In total, 13.64 % of these were captured between 0600 and 0900 h, 54.54 % between 0900 and 1200 h, 22.73 % between 1200 and 15 h and 9.09 % between 1500 and 1800 h. Except for one *A. exotica* found between 0900 and 1200 h, all remaining individuals were *D. clavata* and pollen smears were present on the upper side of the thorax of only one of these, indicating a previous visit to another flower. Functional measurements of both flowers and pollinators are available in Supplementary Data Table S1.

We found that as pollinators walk on flowers, they make abdomen movements consistent with oviposition behaviour (Supplementary Data Movie S1). *Allograpta exotica* usually first hovers in front of flowers, sometimes landing on sepals and lateral petals (Fig. 2A). Then, it lands on the labellum border and starts to move through the opening hole towards the region of the infolded lobes (Fig. 2B–E). When compared to *D. clavata*, it generally landed quicker and more directly on this area.

We found that syrphid flies laid their eggs on several external flower parts including the petals, staminode and labellum (Fig. 3A–I). In the labellum, eggs were found in the external structure of the pouch, around the entrance and in the infolded lobes where the dark spots are located. We also found eggs laid internally on the labellum posterior zone where dark spots were also present (Fig. 3J–K). We also found some hatched first-instar larvae (Fig. 3K). However, we never found any aphids on flowers.

Pollinator effectiveness

We found that male reproductive success ranged from 33.55 to 56.72 % across the four years of the survey, with a total average of 38.44 % (Table 1). One anther pollen removal (30.42 %) occurred more frequently in flowers than two anther removal (8.02 %) (Table 1). Pollen smear removal from two anthers indicates that some flowers were visited by hoverflies at least twice. On the other hand, female success ranged between 13.43 and 20.50 %, with a total average of 16.05 %.

Breeding system

No fruits were formed from unmanipulated or emasculated flowers, but both self- and cross-pollination led to fruit production (Table 2). *Phragmipedium vittatum* was found to be self-compatible, presenting an ISI of 0.04 (<0.8). There were overall differences among the treatments ($\chi^2 = 27.56$, d.f. = 2, $P < 0.001$). Fruit set for open-pollination was less than that for both manual cross- and self-pollination, indicating pollen limitation. However, we did not find pollen limitation of fruit set according to the PL index since the population had a value of 0.64 (<0.8).

Seed number estimates varied widely, ranging between 25 650 and 101 460. We found significant differences in the number of seeds across pollination treatments ($\chi^2 = 12.74$, $P = 0.002$; Table 2). Cross-pollinated fruits had, on average, 54 % more seeds than self-pollinated fruits. However, there were no differences between open-pollination and the two hand-pollination treatments. We also found significant differences in seed viability ($\chi^2 = 36.94$, d.f. = 2, $P < 0.001$; Table 2). All groups were significantly different from each other. On average, the number of viable seeds of cross-pollination fruits was approximately twice that of self-pollination, and the number of viable seeds of open-pollination fruits was around twice that of cross-pollination.

Histological and histochemical analysis

The region where the two infolded lobes of the labellum are joined forms a vertical slipping zone with the presence of characteristic dark spots. Through SEM it was possible to

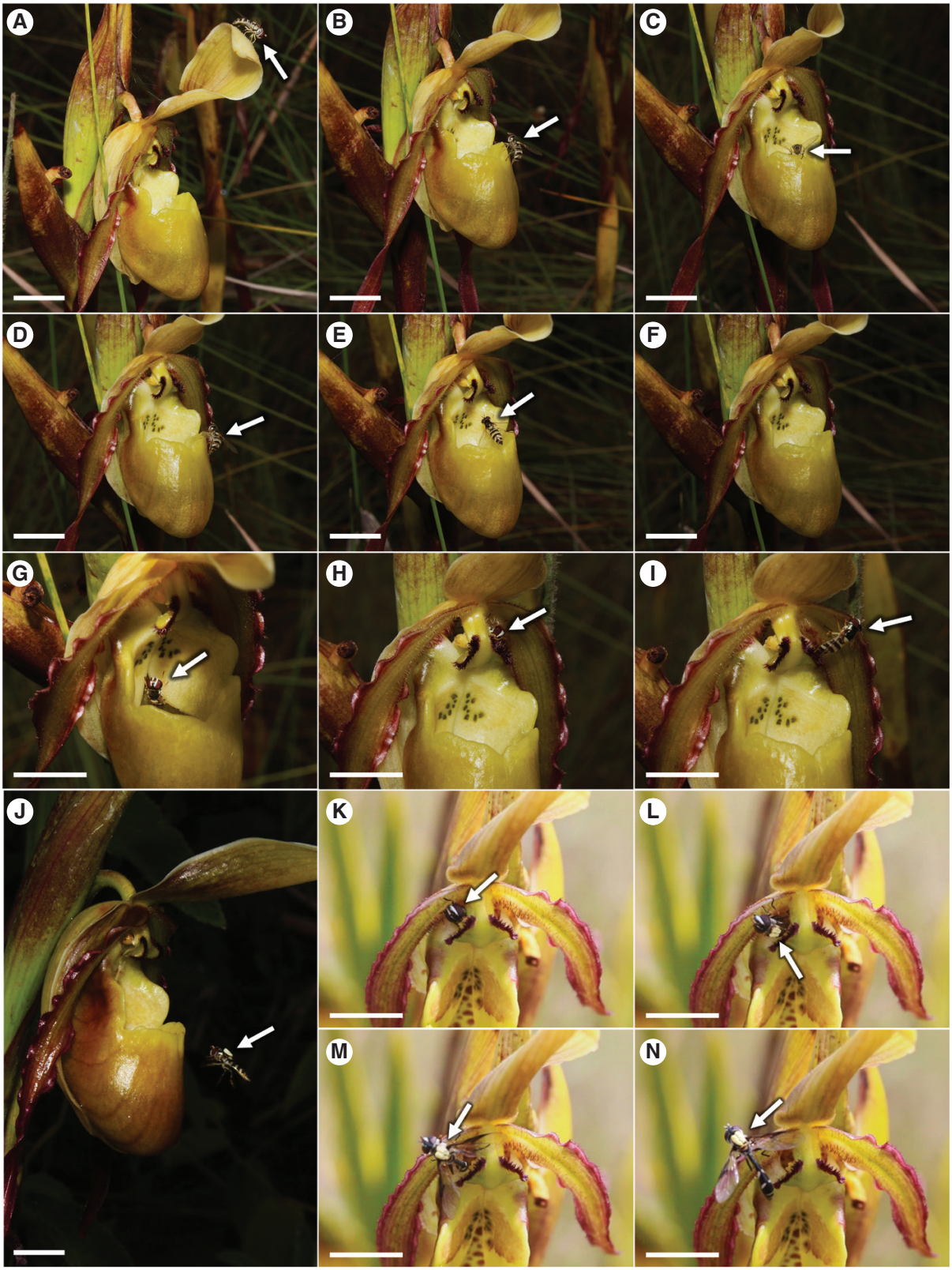

FIG. 2. Pollination process of *Phragmipedium vittatum*. (A–D) *Allograpta exotica* walking through the flower until it (E) reaches the slipping zone and (F) falls in the labellum pouch. (G) *Allograpta exotica* trying to get out by the pouch entrance and (H–I) effectively leaving by one of the exit holes (pollen removed by a previous pollinator visit). (J) *Allograpta exotica* arriving on a flower carrying pollen smears on its upper thorax. (K) *Dioprosopa clavata* trying to leave by one of the exit holes, grabbing the hairs from the petal. (L–N) *Dioprosopa clavata* leaving the exiting hole with pollen smears attached to its upper thorax. Arrows indicate pollinators. Scale bars = 1 cm.

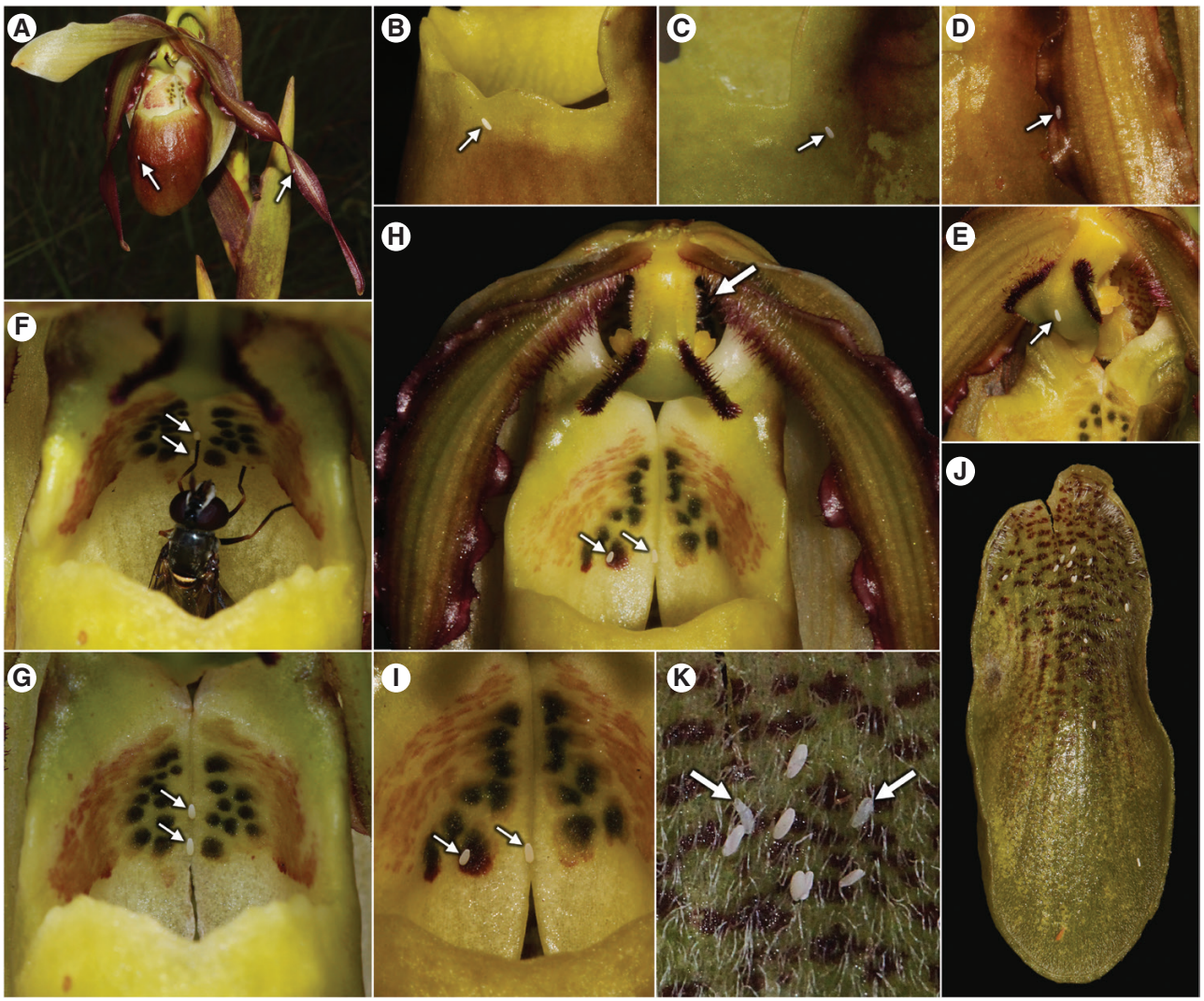

FIG. 3. Egg-laying patterns by syrphid flies in *Phragmipedium vittatum*. (A) Eggs seen from distance on a flower. Eggs laid in the (B, C) labellum, (D) petals and (E) staminode. (F) *Dioprosopa clavata* trying to escape after falling on the pouch and (G) falling back again after depositing the eggs in the spots. (H) Eggs laid on the dark spots with detail of the pollinator inside seen in the right exit hole (larger white arrow). (I) Closer view of the same eggs in H. (J, K) Eggs laid in the inner part of the flower on the dark spots at different magnifications. Arrows indicate eggs, except in K where they indicate hatched larvae.

TABLE 1. *Total number of* Phragmipedium vittatum *flowers surveyed and percentages of male and female pollination success across four flowering seasons.*

Year	No. of flowers	Male success (%)			Female success (%)
		One anther	Two anthers	Total	
2016	306	32.03	8.17	40.20	15.03
2017	161	31.06	5.59	36.65	20.50
2018	301	27.24	6.31	33.55	15.28
2020	67	35.82	20.90	56.72	13.43

identify that this region has downward projecting papillate cells displayed in rows, forming a ridged micropattern (Fig. 4B, C). In the lower portions where the labellum folds, cells become convex-shaped and arranged in a homogeneous pattern (Fig. 4D). There are shape differences in the epidermal cells of the slipping zone, forming a gradient of relief between the dark spots and the other cells surrounding them (Fig. 4G). Such dark spots are formed by cells that group together and, in addition to having different coloration, are taller than the surrounding cells and digitiform, forming high-relief punctuations (Fig. 4E–G). At this same frontal region, we found a positive reaction with the neutral red, indicating high metabolic activity (Fig. 4L). We also found a reaction with ruthenium red, indicating the presence of mucilage (Fig. 4M). We then confirmed that mucilage is produced and secreted by the epidermal cells, as indicated by the positive reaction with PAS (Fig. 4N). As the pollinator reaches this slippery region, probably attracted by the dark spots, it loses its hold and falls into the labellum pouch (Fig. 2E, F; Supplementary Data Movie S1). Both the orientation of downward projecting papillate cells of the epidermis and mucilage secretion probably increase slipperiness such that the animal cannot gain purchase with its feet.

TABLE 2. *Effects of controlled pollination treatments on measures of fecundity in* Phragmipedium vittatum. *Fruit formation indicates the percentage of fruits developed ± s.e. values. Sample size is indicated in parentheses. Number of seeds and seed viability proportion indicate mean ± s.d. values. Different letters indicate statistically significant differences. Emasc. = emasculation; Spont. self- = spontaneous self-pollination.*

	Emasc.	Spont. self-	Self-pollination	Cross-pollination	Open-pollination
Fruit formation % (sample size)	0.00 (17)	0.00 (21)	84.62 ± 0.10[b] (13)	88.24 ± 0.08[b] (17)	31.51 ± 0.05[a] (72)
Seed number	–	–	50 825 ± 17 212[a]	74 423 ± 13 445[b]	61 687 ± 15 283[a,b]
Seed viability	–	–	0.12 ± 0.12[a]	0.32 ± 0.20[b]	0.61 ± 0.11[c]

The basal part of the labellum constitutes a slipper-shaped pouch (Fig. 4A). The posterior part of the entrance hole margin is constituted by the infolded lobes which are formed by a smaller portion of the parenchyma that gradually thickens (≈22 cell layers) towards the anterior region where the outside of the labellum folds into the pouch forming a thicker tissue (that forms a hollow space between the two portions of the labellum) (Fig. 4H). Both outer and inner (arrow) surfaces are covered by the same uniseriate and simple epidermis (Fig. 4H). Internally it forms a thin and slippery surface that prevents the hoverfly from getting out, causing it to fall repeatedly when trying to escape via the entrance hole (Figs 2G and 3F; Supplementary Data Movie S2).

The interior of the pouch is covered by tectorial trichomes, except in the front wall near to the entrance. The bottom where the pollinator falls has shorter trichomes of —three to five cells (Fig. 4A, I). The posterior wall of the pouch has upward oriented trichomes (Fig. 3A) that gradually increase in number and size (approximately six to 12 cells) from the bottom to the top (Fig. 4A, J). The trichomes have a thick cuticle that stained with Sudan red (Fig. 4O). To get out, pollinators need to climb through this posterior portion of the labellum. The progressive increase in the size of these hairs, their upward orientation and the stickiness provided by the cuticle probably helps the pollinator to cling on. This labellum posterior zone also has some dark spots similar to those of the slipping zone (Fig. 4A) that gradually increase in density and apparently form a path that guides the pollinator together with the hairs. The posterior region of the pouch also has a lateral constriction that funnels their passage to the central region (where bigger hairs and spots are located) (Fig. 4A). When reaching the top of the labellum, the pollinator must squeeze itself between the posterior wall and the stigma located below the staminode in a way that pollen smears present on its upper thorax remain adhered on the minute papillae of the stigmatic surface. The staminode that is positioned at the tip of the column has a flattened shape that blocks the base of the labellum and creates two basal lateral apertures. The anthers are also laterally positioned, just before the exit point. When a pollinator squeezes through the gap, pollen is deposited onto its upper thorax by the respective (right or left) articulated anther (Supplementary Data Movie S3). The exit hole is covered by long and abundant hairs from the lateral petals that are grabbed by the pollinator and help it to push its way out (Fig. 2H, K–N). After leaving, some pollinators immediately fly away while others may still spend some time on the inflorescence cleaning themselves. If the hoverfly is deceived again and becomes trapped by a flower on a different plant, it will perform cross-pollination (Fig. 2J).

Neither of the tissues reacted positively with the Fehling reagent, Lugol, Bromophenol blue or NADI reagent. Based on our anatomical and histochemical analyses, we did not find any elaiophores (i.e. specialized oil secretory glands) or nectaries in histological sections, indicating the flowers are rewardless.

DISCUSSION

The results of this study show that *P. vittatum* exploits female hoverflies as pollinators through the combination of floral mimicry and a complex floral trap. We describe several floral traits that are deployed for attraction, trapping and guiding pollinators to the exit. We offer evidence that *P. vittatum* deploys aphid-like cues that deceive female hoverflies searching for oviposition sites to supply their aphidophagous larvae. We also show that *P. vittatum* is rewardless and is a pollinator-dependent self-compatible species that experiences pollen limitation of fecundity. Below we discuss our findings and their implications in detail.

Hoverfly pollination via food and brood-site mimicries is well known from the related *Cypripedium* and *Paphiopedilum* (Bänziger *et al.*, 2012; Pemberton, 2013; Edens-Meier *et al.*, 2014; Jiang *et al.*, 2020; Zheng *et al.*, 2022). While pollination by hoverflies has been documented previously in *Phragmipedium pearcei* (Pemberton, 2013), ours is the first study to confirm that female syrphids lay eggs on flowers of a *Phragmipedium* species (but see Morales, 2017). Such egg-laying behaviour provides clear evidence for a system of oviposition-site mimicry in *Phragmipedium*. Females of many syrphid species deposit eggs around colonies of the aphids on which their predatory larvae feed (Schneider, 1969; Rojo *et al.*, 2003; Almohamad *et al.*, 2009). During their searching behaviour, syrphid females use several cues including visual, olfactory, gustatory and tactile ones (Almohamad *et al.*, 2009). The elevated spots on the *P. vittatum* labellum seems to mimic aphid agglomerations and the respective cues they emit, exploiting the preferences of the female pollinators. The visual resemblance and the behaviour of pollinators are among the main indication of oviposition-site mimicry, with egg-laying providing hard evidence that females have been effectively duped by flowers (Urru *et al.*, 2011; Johnson and Schiestl, 2016).

Aphid mimicry is known only for two orchid lineages: in the Cypripedioideae and in *Epipactis* in the Epidendroideae. Although several factors are responsible for triggering

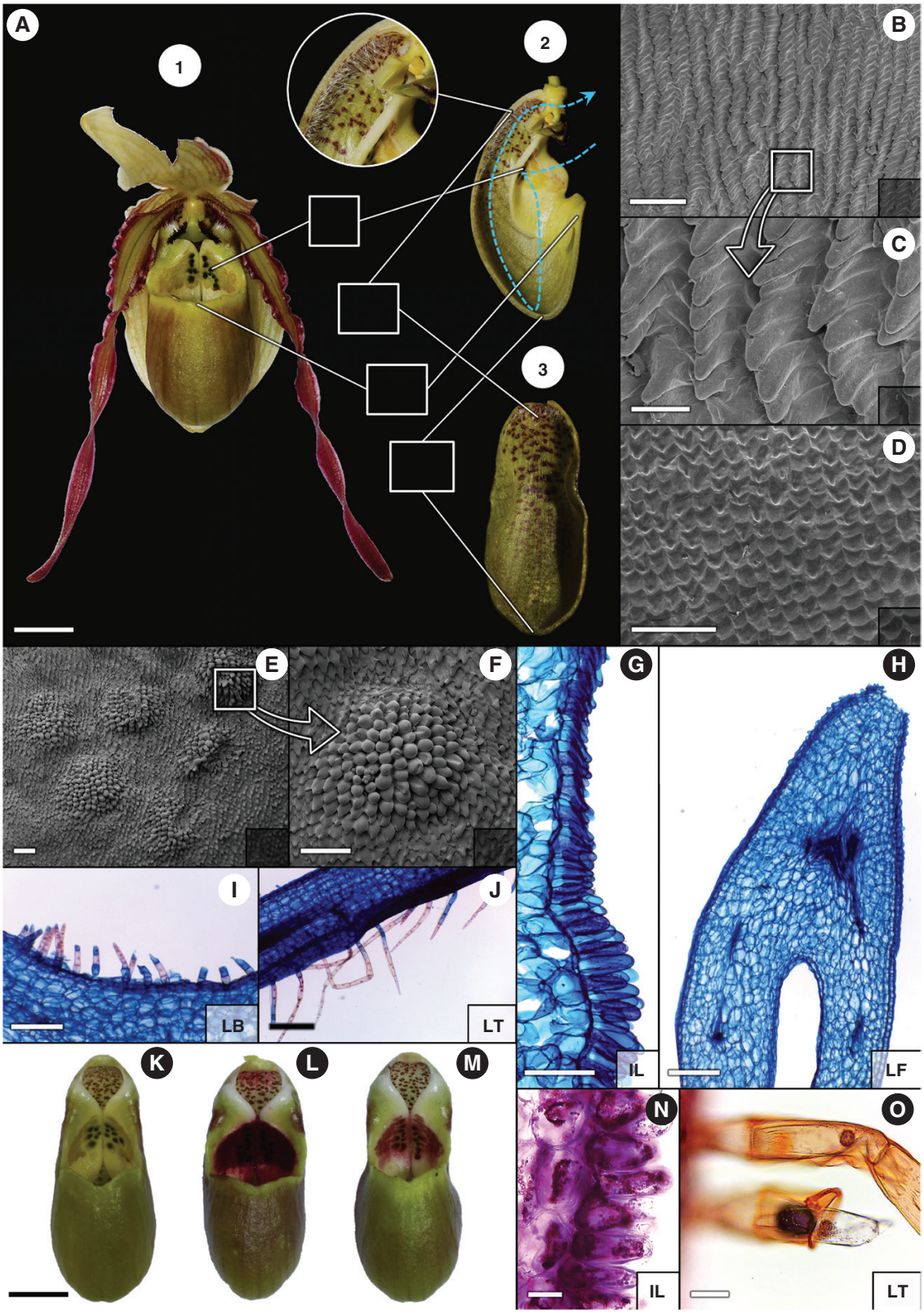

FIG. 4. Floral morphology of *Phragmipedium vittatum*. (A) Frontal view of a flower (1), and lateral (2) and frontal (3) views of the labellum sectioned. The blue dotted line in 2 indicates the route traced by the pollinators. The regions of the flower are specified as: IL, infolded lobes; LF, labellum front; LT, labellum top; LB, labellum base. These are specified at the bottom right of the subsequent images. (B, C) Scanning electron micrographs of the IL in the slipping zone of the IL at different magnifications. (D) Transition between the slipping zone in the lower part of the IL. (E, F) Scanning electron micrographs of the dark spots in the IL at different magnifications. (G) Anatomy of a spot in the IL. (H–J) Anatomy of LF, LB and LT, respectively. (K) Normal labellum followed by (L) neutral red- and (M) ruthenium red-treated labella. (N) Periodic acid-Schiff's-positive reaction on the IL. (O) Sudan red-positive reaction on the trichomes of the TL. Scale bars (at bottom left): A, K, L, M = 1 cm; B, D, E, F, H, I, J = 200 μm; C, G, N, O = 50 μm.

oviposition behaviour in female hoverflies, olfactory cues are among the most important (Almohamad *et al.*, 2009; Stökl *et al.*, 2011) and are likely to be responsible for triggering egg-laying behaviour on both *Paphiopedilum* (Bänziger *et al.*, 2012) and *Phragmipedium.* Some *Epipactis* have black callus-like swellings on the labellum hypochile that are thought to imitate aphids and induce egg-laying (Ivri and Dafni, 1977; Jin *et al.*, 2014; Srimuang *et al.*, 2018). Stökl *et al.* (2011) showed that *Epipactis veratrifolia* flowers emit a mixture of volatile compounds similar to the alarm pheromones released by several aphid species. They also showed via electrophysiological experiments that such compounds are detected by the antennae of hoverflies and that a synthetic mixture of four compounds (produced by the flowers) increased egg-laying by hoverflies.

In *Phragmipedium*, the slipping zone consists of a flat surface formed by the infolded lobes of the labellum where the aphid-like spots are located. While in *Paphiopedilum* spots seem to be elevated and shiny warts, in *Phragmipedium* they visually did not appear to be elevated. However, here we show that in addition to the colour change, there is an increase in the relief of the spots that is distinguishable under magnification. Since syrphid females can use the shape of aphids as a visual cue (Almohamad *et al.*, 2009), such relief may be noticed by hoverflies when they come closer and be important in attraction. In addition, syrphid females prefer to oviposit on colonies with a higher number and density of aphids (Almohamad *et al.*, 2009; Nelson *et al.*, 2012). Thus, the occurrence of several spots simulating an agglomeration pattern may also be an important attractant, which mimics a suitable food supply for larvae and elicit their landing and oviposition behaviours. Colour is also an important cue, and the yellowish tones of several colour parts of *P. vittatum* may additionally function as an attractant, since hoverflies have an innate preference for yellow colours of flowers (e.g. Neimann *et al.*, 2018; Rodríguez-Gasol *et al.*, 2019). Finally, aphid honeydew is a potential attractant as syrphid females use it as a food source (Schneider, 1969) and also as gustatory cue, triggering their oviposition behaviour (Almohamad *et al.*, 2009; e.g. Budenberg and Powell, 1992). Thus, as mucilage constitutes a hydrogel (Røn *et al.*, 2016), it is likely that its presence in the slipping zone also plays a role in pollinator attraction, probably mimicking aphid honeydew.

After pollinator attraction, flowers must effectively trap flies to proceed with the pollination process (Fig. 1). Generally, insects have two attachment devices on their forelegs: claws allow them to cling on to rough surfaces and adhesive pads on to smooth surfaces (Beutel and Gorb, 2001). Falling depends on nullifying these adhesive devices of insects. The presence of downward projecting papillate cells in the infolded lobes of the epidermis of *P. vittatum* provides new insights into the floral micro-morphology characteristics that contribute to pollinator entrapment within the pouch. Although our study is the first to report downward papillate cells in Orchidaceae, this is the most common trait that causes slipperiness in trap flowers, having evolved independently several times (Poppinga *et al.*, 2010). This specific cell shape boosts slipperiness because it is unsuitable for claw anchorage via hooking (Poppinga *et al.*, 2010).

Another important trait we describe is the mucilage secretion in the slipping zone. Surfaces with a liquid film are thought to reduce adhesiveness by preventing the attachment of adhesive pads, leading to an 'aquaplaning' effect (Bohn and Federle, 2004). As mucilage is a viscose and polysaccharide-rich hydrogel, it has lubricating and slippery properties (Røn *et al.*, 2016), which may be important in promoting slipperiness in *P. vittatum* via aquaplaning. In addition, papillate cells are known to play an important role in surface wettability, making surfaces superhydrophilic because water naturally spreads throughout the area (Koch *et al.*, 2008). Thus, the papillate cells already mentioned can also improve mucilage permanence and create a permanent wet surface. As far as we know, we provide here the first report of mucilage as a potential strategy to create slipperiness in insect trapping plants. As mucilage helps the plant to avoid tissue desiccation, other secondary effects may involve the maintenance of cell turgor and shape, especially the elevated region of aphid-like spots and the maintenance of a typical shiny/wetted surface that resembles aphid exudate accumulation.

In *Trigonidium obtusum* and *Coryanthes*, the waxy surfaces are thought to promote slipperiness (Singer, 2002; Gerlach, 2011). In *Cypripedium calceolus*, there are tabular epidermal cells with idioblasts that are thought to prevent claw interlocking (Poppinga *et al.*, 2010), in addition to undetermined fatty liquids (Daumann, 1968) hypothesized to contaminate the adhesive pads of insects (Poppinga *et al.*, 2010). In *Paphiopedilum*, Besi *et al.* (2021) showed the presence of trichomes in the staminode (the slippery region in this genus) of three species (*P. barbatum*, *P. callosum* and *P. niveum*). Despite these observations, knowledge about slipperiness traits in orchids and, more specifically in the Cypripedioideae, is scarce. Here we provide new evidence on which traits mediate pollinator falling via anti-adhesive micro-structural strategies. The combination of several anti-adhesive strategies is thought to increase slipperiness (Poppinga *et al.*, 2010). Thus, downward projecting papillate cells and mucilage secretion on the vertical surface of the labellum lobes are adaptations that can lead to falling of pollinators into the pouch via disruption of both claw interlocking and adhesive pads, respectively.

After being trapped, insects follow a specific and predetermined route within the floral trap structure (Fig. 1). Although the hoverflies try, they cannot get out from the pouch through the entrance because they keep slipping back from the surface where the labellum folds inward. This region has convex-shaped cells that are also thought to prevent claw interlocking (Poppinga *et al.*, 2010). From inside the flower, the light 'window' at the top of the flower makes this region brighter, probably attracting the attention of the insect, which moves up. Since flies are positively phototropic, illumination is used by several trap flowers to guide the pollinators in the intrafloral space to reach reproductive organs and the exit. This may be achieved by window panes, colourless and translucent regions surrounded by areas with darker pigmentation, where light from the outside can easily enter (Faegri and Van der Pijl, 1979; Dafni, 1984; Endress, 1996). Window panes have been reported in other orchid trap flowers (Lehnebach *et al.*, 2005), including lady's slipper orchids of the genus *Cypripedium* (Sugiura *et al.*, 2001; Szlachetko *et al.*, 2020). In *P. vittatum*, although it does not occur clearly at the top of the flower, it is possible to notice areas with less pigmentation in the middle of the labellum where the infolded lobes fuse, extending to the areas surrounding the exit holes. In agreement, these regions

coincide with the path pollinators go though, probably serving as a lighting guide.

It is known that hairs may promote routes of exit (Faegri and Van der Pijl, 1979). Hairs in the posterior part of the labellum seem common in the Cypripedioideae, being reported in other *Phragmipedium* (Pemberton, 2013), *Cypripedium* (Sugiura *et al.*, 2001; Szlachetko *et al.*, 2020), *Paphiopedilum* (Shi *et al.*, 2009) and *Selenipedium* (Cribb and Schiuteman, 2015; Szlachetko and Kolanowska, 2016). In *P. vittatum* we observed that these structures are non-secretory. Such upward oriented hairs progressively increase in size as the pollinator manages to go up, probably serving as a 'stairway' for pollinators to hold on to, aiding their grip by grabbing the longer trichomes during climbing. In addition, the thick cuticle of these hairs may prevent desiccation and make them more resistant to being grabbed.

The dark spots found in the labellum suggest that flowers use aphid colony imitation to exploit the pollinator sensory system even after trapping. The eggs found on the labellum surface corroborate this, indicating use of the same oviposition-site mimicry mechanism to entice pollinators to get in and get out of the pouch. Such inner spotting is also found in other lady's slipper orchids pollinated by hoverflies (e.g. Ren *et al.*, 2011; Jiang *et al.*, 2020).

In oviposition-site mimicry systems, insects tend to move around haphazardly looking for oviposition sites, which may not lead to efficient pollen transfer (Johnson and Schiestl, 2016). Thus, the use of traps, chambers and narrow exit passages allow plants to manipulate pollinators into a precise position for pollen placement (Johnson and Schiestl, 2016). In agreement with this, the set of features reported here, such as less pigmented regions that allow light passage, upward oriented hairs becoming progressively longer, inner aphid-like spotting and lateral constriction of the labellum, forces the pollinator to walk narrow intrafloral paths. Thus, although *P. vittatum* flowers are relatively large compared to their pollinators, they nevertheless achieve precise pollen deposition onto the dorsal part of the thorax of the hoverflies.

After pollen deposition by one of the anthers, the pollinator must keep following its course (Fig. 1). At this point, as the pollinator is closer to the exit, light entering by the exit holes becomes more intense, guiding its way out carrying pollen smears. Another trait that is related to pollination biomechanics is the presence of hairs coming from the lateral petals surrounding the holes. Such petal hairs projecting towards the exit opening holes are also present in *Cypripedium* (Sugiura *et al.*, 2001; Szlachetko *et al.*, 2020), *Paphiopedilum* (Shi *et al.*, 2007) and *Selenipedium* (Szlachetko and Kolanowska, 2016). We observed that as the pollinator holds these hairs, they also may increase anchorage and allow the pollinator to get out through the tight hole. In fact, we found that some hoverflies may get stuck in the exit hole and even die (J.C.F.C., personal observation), indicating that the absence of such hairs would make this more common. After finally leaving, pollinators carry pollen smears onto their upper thorax and will cross-pollinate flowers if they are deceived again by a flower on a different plant where the whole three-phase cycle starts again.

We found that *P. vittatum* is a rewardless, self-compatible, non-apomictic and pollinator-dependent species. Both artificial self- and cross-pollination successes led to high fruit set, demonstrating that there is no self-incompatibility mechanism. However, we found lower levels of seed viability in artificially selfed fruits when compared to both artificial cross- and open-pollination, suggesting some inbreeding depression. Furthermore, total seed numbers after selfing were lower when compared to cross-pollination. Thus, self-fertilization apparently brings deleterious effects, and our results highlight the importance of pollinator-mediated crossing in seed quality and quantity.

Although some Cypripedioideae are autogamous, including some *Phragmipedium* (e.g. *P. reticulatum*, *P. lindenii*, *P. boisserianum* and *P. longifolium*) (Pemberton, 2011, 2013; Edens-Meier *et al.*, 2014; Morales, 2017), *P. vittatum* is dependent on biotic pollination. Studies of *Phragmipedium* reproductive biology are scarce, but evidence shows that natural levels of fruiting success vary, with *P. besseae* having only 4.3 % (Edens-Meier *et al.*, 2014), *P. longifolium* having 12 % (Morales, 2017), *P. pearcei* having 50 %, and the autonomous selfers (*P. reticulatum* and *P. lindenii*) showing 100 % (Edens-Meier *et al.*, 2014). Our study species *P. vittatum* showed an intermediate value of 31.51 % for fruiting success exclusively from pollinator visits.

Across the four flowering seasons, removal of pollen smears from one anther occurred in 30.42 % of flowers while removal from two anthers occurred only in 8.02 %. Although there is the possibility of pollinators exiting by the same hole more than once, this indicates that multiple pollinator visits to a flower are less frequent. In addition, deposition of pollen smears is much less frequent than removal, indicating that most flies do not visit flowers twice (i.e. removing and not depositing the pollen) and probably arrive at flowers without carrying pollen loads, as supported by our analysis of flies captured by blocking the exit holes. Pollen removal and deposition appear to vary across flowering seasons. Although we had a lower PL index when we surveyed fruits (0.64), the analysis of pollen deposition using a larger sample size and a wider period showed lower values of pollination success (16.05 %). This difference could reflect an effect of sample size or, more probably, that pollination success varies over time. Deceptive flowers usually have a lower pollination success when compared to rewarding flowers (Tremblay *et al.*, 2005), but some deceptive species with effective mimicry strategies can have relatively high pollination success (Jersáková *et al.*, 2006).

CONCLUSIONS

Phragmipedium vittatum apparently employs oviposition-site mimicry as flowers have aphid-like spots that deceive female hoverflies with aphidophagous larvae into egg-laying behaviour. It has a sophisticated trapping floral mechanism that ensures effective pollen transfer by the syrphid flies. Traps are basal and widespread in the Cypripedioideae and function to manipulate pollinators into a position where they are squeezed against the stigmas and one of the anthers and in that way the pollen smear can be transferred. This study sheds new light on the sophisticated micro-morphological adaptations that are deployed by flowers of Cypripedioideae to exploit and manipulate their pollinators.

SUPPLEMENTARY DATA

Supplementary data are available online at https://academic.oup.com/aob and consist of the following. Figure S1: *Phragmipedium vittatum* in a Vereda swamp. Table S1: functional morphological trait measurements of *Phragmipedium vittatum* flowers and its pollinators. Movie S1: *Allograpta exotica* reaching the slipping zone and falling into the labellum pouch. Movie S2: *Allograpta exotica* trying to escape by the entrance hole and slipping back. Movie S3: *Dioprosopa clavata* squeezing itself through one of the exit holes while pollen is deposited onto its upper thorax by the articulated anther.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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