The sweet path of Hansel and Gretel: pollination system of Masdevallia hortensis Luer & R. Escobar (Orchidaceae: Pleurothallidinae) in a cloud montane forest of the Cordillera Occidental, in Colombia

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Abstract

Pollination by deception is assumed as the general rule among pleurothallid orchids. However, considering the exceptional diversity of these orchids (44 genera and over 5100 species) and the relatively limited number of available studies (pollination ecology has been assesed in only 17 genera), generalized trends about their pollination systems might disregard a wide variety of specific life-history traits and inconspicuous honest signals/rewards for pollinators. Known associations of pleurothallid orchids with a large assortment of fly taxa further support this assumption. We investigated the natural pollination system of *Masdevallia hortensis*, a strictly endemic species of cloud forests in the Western Andes of Colombia. *Masdevallia hortensis* exhibited a sophisticated and customized pollination mechanism, producing sugary secretions in the lateral sepals along purple dotted patches, fed upon by different visiting species of fruit flies (Drosophilidae). The sucrose concentration in these secretions varied throughout the day and was significantly lower after removing the pollinaria. Visiting fruit flies appeared to be guided towards a chamber between the mobile lip and the column by the dotted lines in the lateral sepals. During visitations, individuals of the most abundant species in our observations (Drosophilidae sp. 1 [AO]) were singly entrapped in the chamber until eventually freeing themselves with the pollinaria attached to their bodies. We also demonstrated that *M. hortensis* is strictly self-incompatible, which makes fly pollination an essential process for the maintenance of natural populations of the species. The flowers of *M. hortensis* offer rewards for visiting insects, an aspect that should also be evaluated among congenerics. In this way, we urge integrative ecological studies to understand the evolutionary patterns of this group of orchids.

INTRODUCTION

In angiosperms, pollination is the successful transference of pollen from a male structure (anthers) to a female structure (stigma) of a plant, leading to fertilization and the formation of seeds (Faegri and Pijl 1979). Animals are the main pollen vectors among flowering plants, and in tropical communities, more than 94% of angiosperms are pollinated both by vertebrates and invertebrates, mostly insects (Ollerton et al. 2011). Plants pollinated by animals exhibit a wide array of strategies to attract and maintain potential pollinator visits, and widespread attraction strategies involve floral advertisement through variation in flower color, color patterns, shape, and scents (Willmer 2011). Rewards implicated in the maintenance of pollination interactions include foodstuffs (like pollen and nectar), meeting and mating possibilities, stable and comfortable temperature (Seymour et al. 2003), shelter, and oviposition resources, among others (Barfod et al. 2011; Renner and Feil 1993; Willmer 2011). Likewise, insects have evolved specialized morphological and behavioral traits. Thus, pollinator perception and its associated behavior constitute key selective elements for floral attributes because they mediate the relationships between floral signals and effectiveness in pollen flow (Schistl and Johnson 2013). Reciprocal evolution of traits such as flower shape, color, scent, even anthesis daytime as well as the pollinators' corresponding receptors, mediate interactions. These interactions are considered to be primary drivers of diversification in some major plant and animal groups (Ollerton et al. 2019; Van der Niet et al. 2014). One of the most exceptional examples of variation in pollinator attractant cues occurs in the family Orchidaceae. Orchids display fascinating visual (e.g. complex combinations of contrasting colors or pollination guides), chemical (e.g. wide diversity of floral volatile organic compounds, or VOCs: Kaiser 1993; Cohen et al. 2021; Hayashi et al. 2021), and morphological traits (e.g. complex mushroom mimicry: Policha et al. 2019) mostly attributed to the attraction of pollinators that range from insects to birds.

Pollination by deception occurs when some plants mimic honest signals, yet do not offer the corresponding reward (Schiestl 2005; Waser and Ollerton 2006; Jersáková et al. 2009). And even though, a recent review gathered new evidence of honest signaling in orchids across subfamilies and tribes that were previously assumed to be outright deceitful (which suggests that the rewardlessness prevalence among these plants might be overestimated: i.e. Shrestha et al. 2020, and information from nearly 2900 orchid species arround the world showed that pollinator attraction is mainly based on rewards i.e. 54%: Ackerman et al. 2023) the idea of rewardlessness as the most common pollination strategy in orchids is widespread (Ackerman 1986; Borba et al. 2011; Johnson and Schiestl 2016; Schiestl 2005). However, it is necessary to increase our knowledge of orchids' pollination systems to understand which strategies may be involved in attracting and keeping visitors. For instance, the flowers of several orchid species produce small quantities of sugary secretions or other compounds (Bogarín et al. 2018; Shrestha et al. 2020), putatively used as food rewards by visiting insects.

With around 5,100 species, the Neotropical subtribe Pleurothallidinae accounts for about 20% of all described orchids (Karremans 2016), and Colombia harbors an exceptional diversity in this group of plants (Karremans et al. 2023) with many species under threat (70% of the Colombian threatened plants are pleurothallids: Ministerio de Ambiente y Desarrollo Sostenible 2020); this makes it urgent to gather basic information about their natural history including reproductive strategies to take in account in conservation actions. Recent assessments of the floral biology and mating systems of pleurothallids have unveiled key synapomorphies that correlate with their floral and/or reproductive traits (Borba et al. 2011; Cardoso-Gustavson et al. 2017; Karremans et al. 2015). Their pollination is mainly attributed to flies (Diptera) and thus often characterized within the frame of 'myiophily' (Borba et al. 2011), a generalistic term that does not adequately encompass the functional ecology diversity of the numerous dipteran taxa as well as the high diversity of plant strategies to attract such a diversity of flies (e.g. Oelschlägel et al. 2015; Raguso 2020), including different groups of flies pollinating pleurothallids (i.e. Karremans and Díaz-Morales 2019). Moreover, some pleurothallids seem to be pollinated by other groups of small insects, such as wasps (Hymenoptera) (Karremans and Díaz-Morales 2019). As a strategy to attract potential pollen vectors, pleurothallids may offer floral rewards, such as nectar, and/or dupe visitors through mate, feeding-site, or brood-site deception (Barbosa et al. 2009; Blanco and Barboza 2005; Borba and Semir 2001; Karremans et al. 2015).

The pollination ecology of pleurothallids has been assessed in less than 2% of the estimated richness for the subtribe (this if pollen removal and flower visitation are considered; Karremans and Díaz-Morales 2019) across 17 of the 44 currently recognized genera (nearly 136 species), including *Acianthera* Scheidw., *Dracula* Luer, *Lepanthes* Sw., *Masdevallia* Ruiz & Pav., *Octomeria* R. Br., *Pleurothallis* R. Br., *Specklinia* Lindl., *Stelis* Sw. and *Trichosalpinx* Luer (Borba and Semir 2001; Albores-Ortiz and Sosa 2006; Blanco and Barboza 2005; Barbosa et al. 2009; Endara et al. 2010; Cuervo-Martínez et al. 2012; Duque-Buitrago et al. 2014; Karremans et al. 2015; Bogarín et al. 2018; Karremans and Díaz-Morales 2019). Therefore, general considerations about pollination systems within the subtribe are tentative at best. Furthermore, floral rewards are not always obvious and can vary considerably across taxa (i.e. nectar, pollen, oils, resins, and volatile fragrances), which might have led to overestimated non-rewarding pleurothallid species (Karremans et al. 2015); in addition, epiphytic orchids, as well as tropical regions in Southern America are under-represented in pollination studies (i.e. Ackerman et al. 2023). Moreover, it is

relevant to stress the remarkable diversity of pollination strategies within pleurothallids. For instance, several fly taxa are reported as pollinators, including Drosophilidae, Calliphoridae, Muscidae, Sarcophagidae, and Sciaridae, among others (Bogarín et al. 2018; Borba and Semir 2001; Karremans and Díaz-Morales 2019; Raguso 2020; Blanco and Barboza 2005). Each fly taxon is associated with a specific 'pollination system', which often involves floral scent signaling (Karremans and Díaz-Morales 2019; Raguso 2020).

With more than 600 species (POWO 2019), the genus *Masdevallia* is the third most species-rich among pleurothallids, exhibiting its highest diversity in the Andes of South America (Abele 2007). Floral visitors have been recorded for 26 species of the genus, but actual removal of pollinia by visitors has only been reported in studies involving seven species (Karremans and Díaz-Morales 2019). Visiting insects are essentially acalyptrate and calyptrate muscomorph flies, notably Drosophilidae, Calliphoridae, and Sarcophagidae (Karremans and Díaz-Morales 2019). These different fly taxa occupy a wide variety of habitats, and many species are well-known generalist flower visitors. Both blowflies (Calliphoridae) and flesh flies (Sarcophagidae) frequently brood and feed on carrion, or in flowers that effectively mimic this resource (Brown et al. 2010; Willmer 2011).

Karremans and Díaz-Morales (2019) described the prevalent trends in pollination mechanisms among pleurothallids, typifying the 'masdevalliform mechanism' for the genus *Masdevallia* and other related taxa. In this mechanism, pollinia removal occurs with a combination of floral features, namely: *i)* wide-open flowers and initial attraction of potential pollinators through fragrances emitted by osmophores on the sepals; *ii)* a movable and elongated lip, which is parallel to the column and exhibits visual or structural guides; *iii)* a ventral stigma, and flattened pollinia or with caudicles, and without viscidium; *iv)* well-centered placement of pollinia on the visiting flies' sub-scutellum, while they exit flowers walking backward. Flower structures' spatial placement allows that when the fly moves towards the movable lip, it is tilted toward the column. These contrast with other mechanisms that do not involve a movable lip or result in the attachment of pollinia to a different body part of the visiting flies (e.g., 'Steliform' and 'Lepanthiform' mechanisms: Karremans and Díaz-Morales 2019), suggesting highly specialized pollinator-mediated selection. What remains unclear for *Masdevallia* is whether the flies are driven to the flower by rewards (honest signaling), or whether this system is entirely deceptive. Evaluating rewardlessness is relevant because it provides a window into fundamental issues of pollination biology (Dafni 1984) and plant-pollinator evolutionary mechanisms (Mitchell et al. 2009). Rewardlessness implies an energetic cost to the plant and may generate energy dependence on pollinators (Johnson and Schiestl 2016). Most importantly, the questions of how and which rewards are offered, and to which potential pollinators, are key for the understanding of both plants and pollinators' natural history and population ecology. Lastly, it is also noteworthy that most pollination studies with pleurothallids have been conducted in semi-open greenhouses or semi-natural conditions (e.g., Cuervo-Martínez et al. 2012), thus influencing the normal behavior of visiting species and arguably disrupting orchid-pollinator relationships. Just as important, valuable information about the structure of the natural community and its effects on pollination dynamics is lost in manipulated scenarios. Detailed knowledge of the reproductive biology of these species in natural scenarios is necessary to propose plans for the management and recovery of natural populations. Pollination is a key process for the maintenance of natural populations and, in general, baseline ecological knowledge is necessary for species-based plant conservation strategies (Heywood 2017). Such information about coexisting species, pollinators, and the use of resources can drive tends in spatial distribution (e.g., Štípková et al. 2020), and can be important for habitat enrichment and the maintenance of natural populations.

In this work, we studied a large natural population of *Masdevallia hortensis* Luer & R. Escobar in a well-preserved cloud forest in the Western Andes of Colombia. We addressed the following questions: (1) how is the pollination mechanism? (2) which are the flower visitors and effective pollinators of *M. hortensis*? and (3) does *M. hortensis* exhibit a deceptive pollination strategy? By answering these questions, we hope to improve the understanding of the pollination biology of this endangered species.

Materials and Methods

Study site — Fieldwork was carried out during separate events on January 2018, April and September 2019, and January and February 2020 (the first two fieldtrips were qualitative and scouting expeditions, and the posteriorly three ones, with systematic measurements; expeditions lasted between five and 15 days) in a cloud montane forest located in the Western Colombian Andes, across the municipalities of Santuario, Apía, and Pueblo Rico (department of Risaralda) in the buffer zone of Tatamá Natural National Park (2,500 m.a.s.l.). The authors refrain from providing exact geolocation due to the vulnerability of natural populations of *M. hortensis* to poaching (Online Resource 1).

Mating experiments and additional observations on plant morphology were conducted in a private greenhouse (Orquifollajes S.A.S.) located in the municipality of Guarne, department of Antioquia, Colombia (2,300 m.a.s.l.).

Studied species — Masdevallia hortensis belongs to the subgenus *Meleagris*, which is characterized by a successively flowering raceme bearing attractive and colorful flowers with widely spreading lateral sepals, free near their bases, forming a shallow cup with the curved shape of the column-foot; the dorsal sepal is ovate, wide, and free from the lateral sepals. The lip is attached to a free extension of the column-foot, and the ovaries of most species are crested (Luer 2003). The species is endemic to moist and shady montane cloud forests of the Western Colombian Andes, at elevations between 2,500–2,600 m.a.s.l. in the departments of Antioquia and Risaralda (Peláez et al. 2006; Vieira-Uribe and Karremans 2017). Although *M. hortensis* is not on the IUCN Red List, natural populations of the species are considered vulnerable (VU) according to the Colombian Ministry of Environment and Sustainable Development (MADS 2020: Resolución 1912 de 2017). A detailed census for the studied population is not provided. Still, we observed the general state and counted each M. hortensis individual found in over 50 phorophytes, and recorded neighboring plant species.

Flowering patterns — We marked reproductive individuals (n = 20) and inflorescences (n = 34), tagging them with an individually assigned code on a small, durable label (developing inflorescences were not taken into account, and therefore were considered vegetative stages during January and February 2020 samplings). Detailed phenological observations took place in September 2019, January, and February 2020 (although we also made non-systematic observations during January 2018 and April 2019). We recorded information such as the number of leaves, the number of scars per inflorescence, and floral stages for each tagged individual. We assigned floral stages to different categories based on the flower aspect and the color of the sepaline tails, as well as

the length of the described aspect. Similarly, we categorized fruits in different development stages to complete the entire phenological description of the reproductive structures. In addition, information about other two syntopic pleurothallid species were recorded in each field expedition with detailed descriptions.

Mating systems — Using cultivated individuals of *M. hortensis*, we conducted three different manipulated pollination treatments: (1) autonomous self-pollination (autogamy): using a single pollinia or a pollinarium from the same flower (n = 30); (2) self-pollination (geitonogamy): using a single pollinia or a pollinarium from different flowers of the same plant (n = 15), and (3) cross-pollination (xenogamy): using a single pollinia or a pollinarium from a flower of a different individual (n = 20). Additionally, some flowers were emasculated and left unpollinated (n = 10 per test), as a test of apomixis (agamospermy). In all treatments, flowers were covered with tea bags to avoid the access of insects.

Floral droplets — The floral secretions that accumulated on the adaxial surface of the lateral sepals were sampled in situ from 27 flowers with disposable microcapillary pipets (Kimble® 71900-20 KIMAX® 20µL Glass Micro Capillary Pipet, TC, Black Color Code, Non-Sterile & Disposable). We collected samples twice a day for two days, once during daytime (10:00 to 13:00 h) and once during nighttime (19:00 to 21:00 h). The sucrose content in the samples was analyzed with a hand-held refractometer (Eclipse Refractometer Sugar °Brix 0–50%). Differences in the sucrose concentration between samples collected during daytime (n = 20) and nighttime (n = 23), as well as between flowers with (n = 30) and without pollinarium (n = 13) were compared by one-way ANOVA using R Studio.

Behavioral observations of potential pollinators — We recorded the behavior of visiting insects on fully open flowers (n = 30) between 06:00 and 18:00 h, totaling ca. 190 hrs. of observations. Still, images and video footage (ca. 1 h) of flowers and insects were obtained with a digital camera coupled with a macro lens (a Nikon D3400, and a Canon Rebel SL1 camera coupled to a Canon® MP-E 65 mm). Changes in the flower column resulting from the activity of flies (pollinia removal or deposition) were observed under 30x magnifying glass and complemented with photographic documentation.

Insect collections and identifications — Flower-visiting insects were captured with the aid of an aspirator or a plastic bag and then transferred to 2 ml Eppendorf tubes containing 70% ethanol. Specimens were morphotyped and identified to the lowest possible taxonomic level using identification keys, original descriptions, and revisions (e.g. Brown et al. 2010; Miller et al. 2017). Collections were conducted under a specific permit issued to Universidad CES (Resolución 0790, 2014, Available at http://portal.anla.gov.co/sites/default/files/13312_res_0790_180714.pdf). Voucher specimens are deposited in the 'Colecciones Biológicas de la Universidad CES' (CBUCES; RNC 209). Most of the insect visitors collections were carried on along the exploratory field trips which allowed us to identify key morphological traits previous to behavioral field observations, for linking the morphospecies to recorded behaviors unambiguously.

We assigned a number for each identified morphospecies (not resolved until species level), along with the name initials for the author of the morphospecific identification. Of note, in most studies, identifications of drosophilid flies involved in pollination are restricted to the genus level, even when taxonomic assessment is assisted by specialists (Endara et al. 2010; Policha et al. 2019). Therefore, while several new insect species await valid descriptions, such a catalog allows us to continue building a consistent nomenclature within a traceable timeframe (i.e., Maia et al. 2021). These temporal names are linked to high-resolution images of the morphospecies and made available through the CBUCES (2020) web page (https://sitios.ces.edu.co/cbuces/#centro_datos_page).

To avoid the loss of external cuticular setae and other important taxonomical traits, fly specimens were submerged in glycerin and then digitally photographed with a Canon Rebel SL1 camera coupled to a Canon® MP-E 65 mm attached to stereomicroscope stand. The images were captured using Helicon Remote software (version 3.9.9 M) and captured images were stacked in Helicon Focus (version 7.6.1 pro) to produce high quality, pan focal images (i.e., Flórez-V et al. *in press*). For efficient light diffusion, a dome was used along with a tracing paper ring, which was placed around specimens (Kawada and Buffington 2016). The final images were edited, and figure plates were composed in Adobe Photoshop® v21.1.0.

Results

Field observations of the natural population — The studied population was comprised of a little more than 150 individuals of *Masdevallia hortensis* patchily distributed in a well-conserved montane cloud forest. The forest had a partially open canopy due to forest glades from fallen trees. Individuals of *M. hortensis* were most abundant along thin horizontal branches of trees or on fallen trees covered by moss and restricted to the mountain ridge, rarely found on the slopes and troughs. Also, we observed *M. hortensis* growing in conspecific aggregations (gregariously) and/or with other pleurothallids. These polyspecific assemblages often included individuals of *Andinia nummularia* (Rchb. f.) Karremans & S. Vieira-Uribe, *A. pilosella* (Rchb. f.) Karremans & S. Vieira-Uribe, *Diodonopsis anachaeta* (Rchb.f.) Pridgeon & M.W.Chase, *Dracula chestertonii* (Rchb.f.) Luer, *D. iricolor* (Rchb.f.) Luer & R.Escobar, *Lepanthes discolor* Luer & R. Escobar, *L. manabina* Dodson, *L. nicolasii* Luer & R. Escobar, *M. amanda* Rchb.f. & Warsz., *M. nidifica* Rchb. f., *Platystele* Schltr., *Pleurothallis* spp., *Scaphosepalum odontochilum* Kraenzl., *S. swertiifolium* (Rchb.f.) Rolfe, *Stelis* spp., *Teagueia phasmida* (Luer & R.Escobar) O.Gruss & M.Wolff., and *Trichosalpinx* spp.

We recurrently observed individuals of *M. hortensis* in association with *L. nicolasii* and *S. swertiifolium* (nearly 70% of the studied phorophytes had the three species coexisting), blooming at the same time. This association was documented in a similar forest patch near the type locality of *M. hortensis* ('vereda el Centello', municipality of Jardín, department of Antioquia), and on the western slope of Tatamá mountain ('vereda Montebello', municipality of Pueblo Rico, department of Risaralda).

Population phenology, flowering patterns, flower structure, and flower longevity — We recorded individuals of *M. hortensis* in nine phenological stages during each of the five field expeditions; floral stages were asigned different categories based on the flower aspect and the color of the sepaline tails (Fig. 1), as follows: (1) immature stage-one: predominantly green-colored, flower bud recently formed (Fig. 1a); (2) immature stage-two: predominantly purple-colored

(Fig. 1b); (3) mature stage: predominantly yellowish to orange-colored, flower bud begins its opening (Fig. 1c); (4) mature stage: fleshy flower with vivid white, purple, yellow and orange colors and receptive to pollination (Fig. 1d); and (5) senescent stage: flower with a darker and dried aspect, with yellow and orange colors on lip and base of sepals turning to a reddish color, ovary is pinky-colored with purple dots, and non-receptive to pollinia (Fig. 1e). The fruits development stages were: (1) immature stage: the space between the persistent sepals is more closed, sepals are shortened and whitish pinky-colored, ovary is elongating and turning lighter (Fig. 1f); (2) intermediate stage: shriveled sepals and light green-colored capsule surface (Fig. 1g); (3) mature stage: capsule completely formed, dark green-colored with darker dots (Fig. 1h); and (4) dehiscent capsule: seeds exposed (Fig. 1i). Moreover, there was a different number of individuals in each phenological stage during the sampling months (Fig. 2a). In September of 2019, the focal plants were chosen based on whether they were in reproductive stages (no sterile plants were tagged). We observed that most individuals were flowering; some were also producing floral buds, and few had already formed capsules (Fig. 2a-b). In January 2020, the sampled plants showed a low production of flowers and most of them were in a vegetative stage (Fig. 2b). Conversely, fruit formation increased in February 2020 and new floral buds were observed, although most sampled plants were also in vegetative stages (Fig. 2b). Through non-systematic observations, we scouted the area in April 2019 and individuals bearing capsules were frequent in the population. Production of leaves, as well as the number of produced inflorescences, did not distinctly change between sampled months (Fig. 2c). The production of floral scars increased mainly between September 2019 and January 2020 (Fig. 2c).

This species produced long-lived and sequentially flowering racemes (see below floral buds' fate when the capsule is growing simultaneously); a maximum of 11 flower scars in our measurements suggested raceme longevity of over one year is possible. Large plants may bear three simultaneously opened flowers, but only one per raceme. Recently formed capsules were always found simultaneously with a floral bud inside the floral bract. However, with the progressive growth of the fruit, the floral bud dried out, with flowering succession arrested after fruit formation. On the other hand, the greenhouse observations as well as the field trips observations, let us to trackg of the growing buds, floral development, floral scars, and immature capsules between September 2019 and January 2020 which allowed us to determine the development time for each structure (tracked buds n = 85; tracked flowers n = 48; and tracked capsules n = 52) and the length of the entire development was calculated through the sum of the mean time of each developmental stage was 172 days (Fig. 2d).

Key floral features – We found floral traits that seem to be directly related to the pollination ecology of *M. hortensis*, based on the behavior of insects caught by the column and lip (see below). These traits include: *i)* sepaline tails that appear to function as a landing track surface for flower-visiting insects (Fig. 3a); *ii)* linearly arranged purple dots along the sepals, which seem to play a visual guide role (Fig. 3b); *iii)* disposition of the column, which is arched and dorsally disposed relative to flower placement, concave, and parallel to the lip, with the stigma on the abaxial surface of the column, inner to pollinaria placement, with the rostellum exposed allowing pollinaria adherence (Fig. 3d); *iv)* movable lip, with purple dots (similar to those in the sepals; Fig. 3d); *v)* petals with a longitudinal dorsal keel and convex surface towards the inner face, which seems to help retain visiting insects laterally (Fig. 3d).

In addition, sepals have marginal trichomes (Fig. 3c), never before described for this species, that are similar to the pubescence described for *Masdevallia segurae* Luer & Escobar (although less densely arranged). In some individuals, we observed that the trichomes can vary from distinctly long to very short (like a cuticular protuberance). Also, as stated in the original description of *M. hortensis*, no trichomes were observed on the sepal surface (as was described in *M. segurae*).

Flower visitors, pollination biology and insect behavior — We collected 36 drosophilid flies across five morphospecies, most of which assigned to Drosophilidae sp. 1 [AO] (21); two weevils belonging to a single morphospecies of the subfamily Baridinae (Curculionidae); and one morphospecies of ant (Table 1 and Fig. 4). Flowers were recorded with visitors predominantly from 8:00–16:00 h; no visitors were observed from 18:00–20:00 h. CBUCES codens: CBUCES-F 7796 to CBUCES-F 7799; CBUCES-F 7807 to CBUCES-F 7808; CBUCES-F 7811; CBUCES-F 7816 TO CBUCES-F 7819; CBUCES-F 7821; CBUCES-F 7824 to CBUCES-F 7825; CBUCES-F 7829; CBUCES-F 7934 to CBUCES-F 7935; CBUCES-F 8770; CBUCES-F 8773; and CBUCES-F 8785g. The dataset will be available through GBIF following Salim et al. (2022) structure or GLOBi (Poelen et al. 2014).

Table 1
Insect visitors to flowers of *Masdevallia hortensis* in the buffer zone of Tatamá Natural National Park.

Order/Family	Visitors	Total individuals
Diptera: Drosophilidae	*Drosophila* sp. 1 [AO]	1
	Drosophilidae sp. 1 [AO] [a, b]	21
	Drosophilidae sp. 2 [AO]	1
	Drosophilidae sp. 3 [AO]	7
	Drosophilidae sp. 4 [AO]	3
	Drosophilidae sp. 5 [AO]	1
Diptera: Culicidae		1
Coleoptera: Curculionidae	Baridinae sp. 1 [AO]	2
Hymenoptera: Formicidae		1
[a]Species for which we recorded individuals that removed pollinaria		
[b]Species with recorded individuals that deposited pollinaria		

Flies arrived at flowers one at a time but gradually gathered to form groups, sometimes of different species, summing up to six individuals. They approached the flowers in an uneven zig-zag flight and landed on the sepaline tails of the lateral and dorsal sepals. Upon landing, they immediately walked to the adaxial surface of the lateral sepals towards the purple dotted lines, where droplets of sugary secretions gathered (see below), and began to inspect with legs and mouthparts from the apex to the base of the sepal. The visiting flies inspected the entire flower but spent most of the time on the lateral sepals, where they licked the sugary droplets until consuming them (Fig. 6a-b). Occasionally, they walked towards the lip and seemed to feed on something from the surface, but we could not unambiguously identify any floral secretions on the lip. When a fly walked to the lip apex, its weight initially maintained the lip downwards (Fig. 6c); however, when the fly approached the lip base, this structure was moved abruptly upwards and, consequently, the fly was slammed against the column. The thorax of the fly was then dorsoventrally trapped between the concave column and movable lip, and on the sides by the petals, which are convex towards the inner surface (Fig. 3d). Additionally, sometimes the dorsal keel of the petals also hindered escape efforts from the trapped fly.

After pollinaria attachment, the loaded fly is released from the columnar chamber through a strong struggle with its legs and then discharged on the lateral sepal by the movable lip. Upon struggling to free itself, the trapped fly removed the pollinarium, which adhered to the sub-scutellum. The fly detaches the pollinarium anther cap by moving its legs and wings, being able to take off thereafter. When the arriving fly was already loaded with a pollinarium, its placement of it over the sub-scutellum aided its deposit on the stigma. The total duration of the aforementioned sequence of events, from initial trapping to pollinarium removal, varied from 30 to 80 min and was performed by Drosophilidae sp. 1 [AO], which was the only fly species that went to the sepal basis and was trapped by the flower (Online Resource 2, Video S1).

Mating system and floral rewards — No flowers from the emasculation or self-pollination treatments (autogamy and geitonogamy) developed capsules. In these treatments, all ovaries were aborted and senescence started two or three days following manual deposition of pollinia. Contrastingly, 75% of flowers in the cross-pollination (xenogamy) developed capsules.

During anthesis, flowers produced a sweet smell, perceptible from 08:00 to 15:00 h, but noticeably the most intense between 10:00–13:00 h. Moreover, flowers produced a secretion along the nearly linear arrangement of purple dots on the lateral sepals (see aforementioned), beginning after the initial opening of the flowers and continuing throughout the day. The secretion was produced as discrete droplets (Fig. 5a) that were collected by surface tension and bulged until eventually completely covering the lateral sepals as large drops (Fig. 5b). When the liquid was collected, the plant produced the drops again. The sugary secretion was consumed by flies, ants, and other floral visitors (Fig. 6). The sucrose concentration of the secretion varied between 0° and 10° Brix and was significantly higher during daytime (Mean ± SD: 4.941 ± 2.171; nighttime: 2.235 ± 1.239; $p < 0.001$, $\alpha = 0.05$) (Fig. 5c). Additionally, the sucrose concentration was lower in flowers with removed pollinaria (Mean ± SD: 2 ± 1.643; non-removed pollinaria: 3.929 ± 2.197; $p < 0.001$, $\alpha = 0.05$) (Fig. 5d).

Discussion

Our *in situ* study of the pollination system of the endangered *Masdevallia hortensis* reveals a sophisticated interaction mechanism with the main pollinator, a drosophilid fly. The intricate pollination process involves initial olfactory and visual attraction, a finely tailored trap that concludes in the removal of pollinia, and reward to the pollinator in the form of a sugary secretion. Therefore, our results provide the first documented case of rewarding flowers in the genus *Masdevallia*, in contrast with the more prevalent rewardlessness strategy across this genus (Karremans and Díaz-Morales 2019). We further recorded other visitors and one pollinator (successful pollinarium remover and depositor), and conducted experiments of mating systems and observation on reproductive stages to estimate the development time of floral stages relevant to the pollination process, showing that *M. hortensis* relies in cross-pollination facilitated by Drosophilidae sp. 1 [AO]. This study is the first to describe in detail the habitat of *M. hortensis*, including information on other coexisting orchid species. Together, our findings in *M. hortensis* deepen the knowledge of pleurothallids' interactions and natural history as supporting information for the conservation of this group.

Systematic phenological observations of the studied population of *M. hortensis* were punctually made across three non-sequential months. However, in addition to our visits to the study site, some botanical records of flowering individuals between April and August (Gallego, pers. comm.), suggest that plants in this particular population could be flowering yearlong. We observed a more pronounced production of floral buds in February and September, indicating a flowering peak during the transition from the dry to the rainy season (April-May and September-October). Phenology descriptions in pleurothallid orchids are very scarce with information in Neotropical ecosystems coming mainly from original species descriptions and few records for *Masdevallia* species (i.e., Matallana-Puerto et al. 2022); in *M. cocciea* in the Cordillera Oriental in Colombia, flowers were also found along the year with flowering positively related to precipitation two months ahead (Matallana-Puerto et al. 2022).

On the other hand, new leaves and inflorescence growth could be determined by the development stage of the current floral axis especially by the cessation of it by withering, herbivory, or fruit production. The number of leaves and inflorescences remained unaltered across sampled months, what is common in monocots with a constant numbre of leaves. The haltering of flowering due to capsule formation happened in other coexisting pleurothallids (*Dracula chestertonii* and *Masdevallia nidifica*; AO-M observation). In contrast, we noted that the two syntopic *Scaphosepalum* species can produce capsules simultaneously with flowers in anthesis, probably related to different behavior of their flower visitors which does not favor efficient pollination of these species (even though this must be assessed with future quantification of reproductive success, during the first three fieldtrips we made observations on *Scaphosepalum odontochilum* and *S. swertiifolium* although their flowers had frequent fly visits, capsules formation was scarce); consequently the plants are forced to maximize their chance of reproduction, by maintaining a flower along with capsule formation.

Self-incompatibility is generalized among the Pleurothallidinae (Borba et al. 2011, although see proportion from Ackerman et al. 2023). Mixed reproductive systems have been reported in *Masdevallia*, from self-compatible (see *M. coccinea* and *M. ignea*: Cuervo-Martínez et al. 2012) to cases where individuals within a population can range from self-compatibility to strict self-incompatibility (see *M. infracta*: Borba et al. 2011). Our results showed that *M. hortensis* is completely self-incompatible, rendering natural populations restricted dependence on pollination services provided predominantly by Drosophilidae sp. 1

[AO]. Therefore, efficient conservation efforts for *M. hortenis* must focus not only on the integrity of individuals of this species in natural areas but also on ecological requirements for the long-term maintenance of viable populations of its highly specialized pollinators.

One of the most important aspects involved in the pollination of *Masdevallia hortensis* is the production of sucrose-containing secretions along purple dotted lines on the lateral sepals, on which the visiting flies feed during the daytime when the sucrose concentration was higher. Although deceit in pollination biology is usually associated with rewardlessness, some species use the deceit strategy to attract visitors, but then provide a reward of nectar in order to keep the visitors on the flowers longer to maximize the probability of pollination (aggregation pheromones: de Melo et al. 2010; Karremans et al. 2015). Furthermore, the extent of deceptive systems in orchids, has been overestimated, and systematically recorded supporting evidence is absent in more studies than previously thought (Shrestha et al. 2020). Recently Ackerman et al. (2023) showed that for tropical species and particularly in South Amirican orchids an important proportion of the species involves rewards for pollinator attraction; they showed a similar pattern for the Pleurothallidinae worldwide.

Honest signaling and floral rewards (i.e., nectar guides that steer pollinators to the cavity between the labellum and the column) have been documented as pollination efficiency 'boosters' among pleurothallids (Borba and Semir 2001; Blanco and Barboza 2005; Barbosa et al. 2009; de Melo et al. 2010; Duque-Buitrago et al. 2014; Karremans et al. 2015; Bogarín et al. 2018; see compiled information in Ackerman et al. 2023). During our observations, the studied population of *M. hortensis* exhibited abundant individuals in different developmental stages and sizes, which can be evidence of a balanced population structure linked to successful reproduction. Furthermore, nectar production along the dotted linear patches on the lateral sepals was higher during the day but persisted through the night, and even though the sucrose concentration is lower, this nectar potentially mantain flies alive by offering a constant resource for lingering visitors; in other plant congeneric species concentration of certain compouns showed to be correlated with pollinator preferences (e.g., Tiedge and Lohaus 2017), and in other plant species the sugar production after nectar removal can increases, then relying pollination in different functional groups (e.g., Amorim et al. 2013).

The gregarious distribution pattern of individuals in the studied population, arguably a characteristic of the species, could perhaps be understood as a trade-off in which plant density enhances competition between individuals whereas facilitating pollen transference among neighboring plants. The floral rewards can appeal to a subset of locally available potential pollinators, which are generalists in feeding, and therefore this floral resource is not unique. Nevertheless, visiting insects are filtered out to a single highly efficient pollinator in a specific mechanical interaction with the flower, in contrast to interaction nets for other plant groups like palms where flower parts could be potentially available to a wide variety of visitor morphotypes (Núñez Avellaneda 2014). In addition, small quantities of sucrose-containing secretions, considered as minute rewards, can lead the pollinators to visit neighboring plants, thereby decreasing outcrossing (Shrestha et al. 2020) and probably leading to nested spatial patterns that in turn could be structuring a subset of pollinator populations.

But what is the identity of the main pollinator? We reported the removal and cross-deposition of pollinarium/pollinaria of *M. hortensis* by Drosophilidae sp. 1 [AO], which was the most collected and observed visiting insect species in flowers, and some individuals were documented and photographed with adhered pollinaria exclusively of *M. hortensis*. Other studies have reported pollination by drosophilid flies among the congenerics *M. coccinea, M. ignea, M. infracta, M. floribunda*, and *M. tuerckheimii* (Cuervo-Martínez et al. 2012; Lipińska et al. 2019), all of which bearing flowers with vibrant colors that vary from pink, dark red and dark purple. On top of these studies, there are some fortuitous observations of flower-visiting drosophilids in other species of *Masdevallia* (see Karremans and Díaz-Morales 2019). Nonetheless, throughout our investigation we did not observe the pollinators of *M. hortensis* visiting the other 17 coexisting pleurothallid species. On the other hand, even though during the taxonomic work it was not possible to identified the main pollinator to genus level, the herein used nomenclarute for morphospecies helps improve basic knowledge about Neotropical pollinators and the understanding of the degree of specificity in insect-plant interactions (see methods above).

Two studies involving three other species of *Masdevallia* interacting with drosophilid flies (although in ex- situ conditions) concluded that their pollination is by deceit, as flowers lack apparent rewards and signals appear to mimic the provision of sustenance or places to hide (Chavarro et al. 2006; Cuervo-Martínez et al. 2012). In a similar manner to *M. hortensis*, purple and red-colored spots can be found on the flowers of the focal species assesed in the aforementioned studies, but in comparison to these studies, we noted the occurrence of the production of sucrose-containing secretion just over these dots on the lateral sepals. Some studies have reported that the purple pigments in flowers are associated with visual signals concomitant with sugar production, possibly via the metabolic route in the biosynthesis of anthocyanidin/anthocyanin flavonoids (Willmer 2011). Our here documented observations could be explained through chemical ecology studies involving not only *M. hortensis* but also other pleurothallid species, which apparently also produce on their sepals (A O-M observation) which could be also rewarding secretions. Interestingly, some flowers produce sucrose-containing droplets that are not immediately consumed and then adhere to form a larger drop or ooze that covers much of the sepal. Liquid surfaces could also act as alternative visual signals, a mechanism that could be evaluated for this species (e.g. light reflection on translucent liquids could increase the detectability of key reproductive structures; see pollination in *Ephedra*: Rydin and Bolinder 2015).

Research to characterize floral volatiles appear to be an obvious next step to better understand the attraction of visiting insects to flowers of *M. hortensis*. Such a study is currently underway (Ospina-M et al. in prep). Further, tests of whether the production of volatiles is restricted to the osmophores (and which is the specific location of these osmophores) or if these are also emitted by the sweet liquid are also relevant to understand this pollination interaction. In addition, the composition of these droplets should be studied, in order to determine whether they contain lipids or proteins, compounds that have been detected in other orchids (e.g. Bogarín et al. 2018).

The 'myiophylous pollination syndromes' are not by any means uniform, and we strongly recommend avoiding the term 'syndrome' since the definition behind this term is a reduced interpretation of the complexity of pollination interactions. Pollination syndromes attempt to link suites of ecological, physiological, and behavioral traits from plants and pollen vectors, which given the astonishing diversity of the interacting groups result in a misinterpretation of more complex patterns in nature. Myophily might be extremely wide; there are nectar-seeking flies (Ephydroidea and Tephritoidea), fungus gnats (Mycetophilidae), various groups of saprophages (e.g. blow flies, Calliphoridae; flesh flies, Saprophagidae), among others (Karremans and Díaz-Morales 2019). The strategies for attracting and maintaining visiting insects can be completely contrasting between *M. hortensis* and the other syntopic, co-flowering pleurothallids. For

instance, *Dracula chestertonii* presents a strong, fungus-like odor and appearance (see Kaiser 2006; Endara et al. 2010; Policha et al. 2019). These diverse strategies may be contributing to reproductive isolation among coexisting species with high niche overlap, preventing hybridization, and reducing pollinator competition by ecological niche segregation. Given the high species richness and neighboring coexistence of pleurothallids in forests, like the scenario depicted in our study, these issues are of particular relevance.

Along the lines of the highly sophisticated and varied forms of pollination in pleurothallids, the system in *M. hortensis* features a highly customized mechanism that depends not only on the flower traits that attract and reward the pollinator, but also on some fly attributes such as cognition, weight, size and its ability to get free from the flower structures resulting in the removal of pollinaria. Cloud forests can be huge, dark, and confusing landscapes where a small pollinator can get lost, despite having highly efficient sight like drosophilids; however, like in Hansel and Grettel story, *M. hortensis* offers a sweet entrance for its pollinator, which also outlines the 'secure' way to trap it, and like the young kids, the pollinator fly finds the way to escape, not without a gift stuck on his sub-scutellum.

The described system opens new questions related to deceptive/rewarding strategies in other *Masdevallia* and other pleurothallids species, and integrative ecological studies will strengthen the natural history knowledge as well as our evolutionary understanding of this group of orchids, knowledge that is also key in conservation strategies targeting this group. Colombia as a highly pleurothallids' diverse country offers fields laboratories to continues unveiling the natural history of these groups of interacting organisms.

Declarations

Author Contribution

The study conception and design were performed by A.O-M., J.C-D and M.J.S. Material preparation, data collection and analysis were performed by A.O-M. and J.C-D. The first draft of the manuscript was written by A.O-M. and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Figures

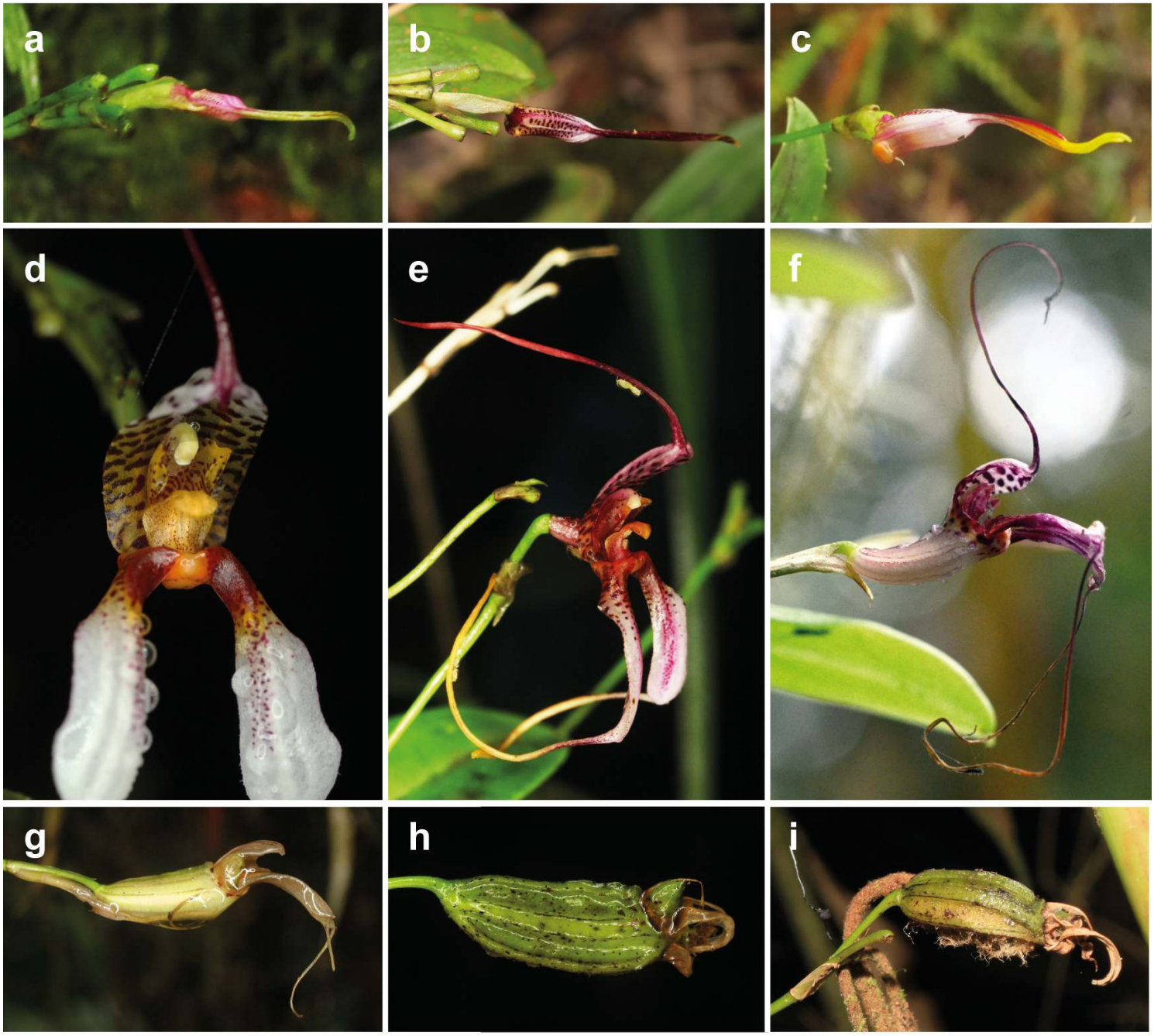

Figure 1

Phenological changes in reproductive structures in *Masdevallia hortensis*: a-c. Floral buds. a. Immature stage-one, flower bud recently formed. b. Immature stage-two. c. Mature stage, floral bud becomes open. d-e. Flower stage. d. Fleshy flower; e. Senescent flower. f-i. Fruit development. f. Immature stage, with persistent sepals. g. Intermediate stage. h. Mature stage. i. Dehiscent capsule, open leaving the seeds exposed. Photographs by Camilo Flórez-V and David Gómez-M.

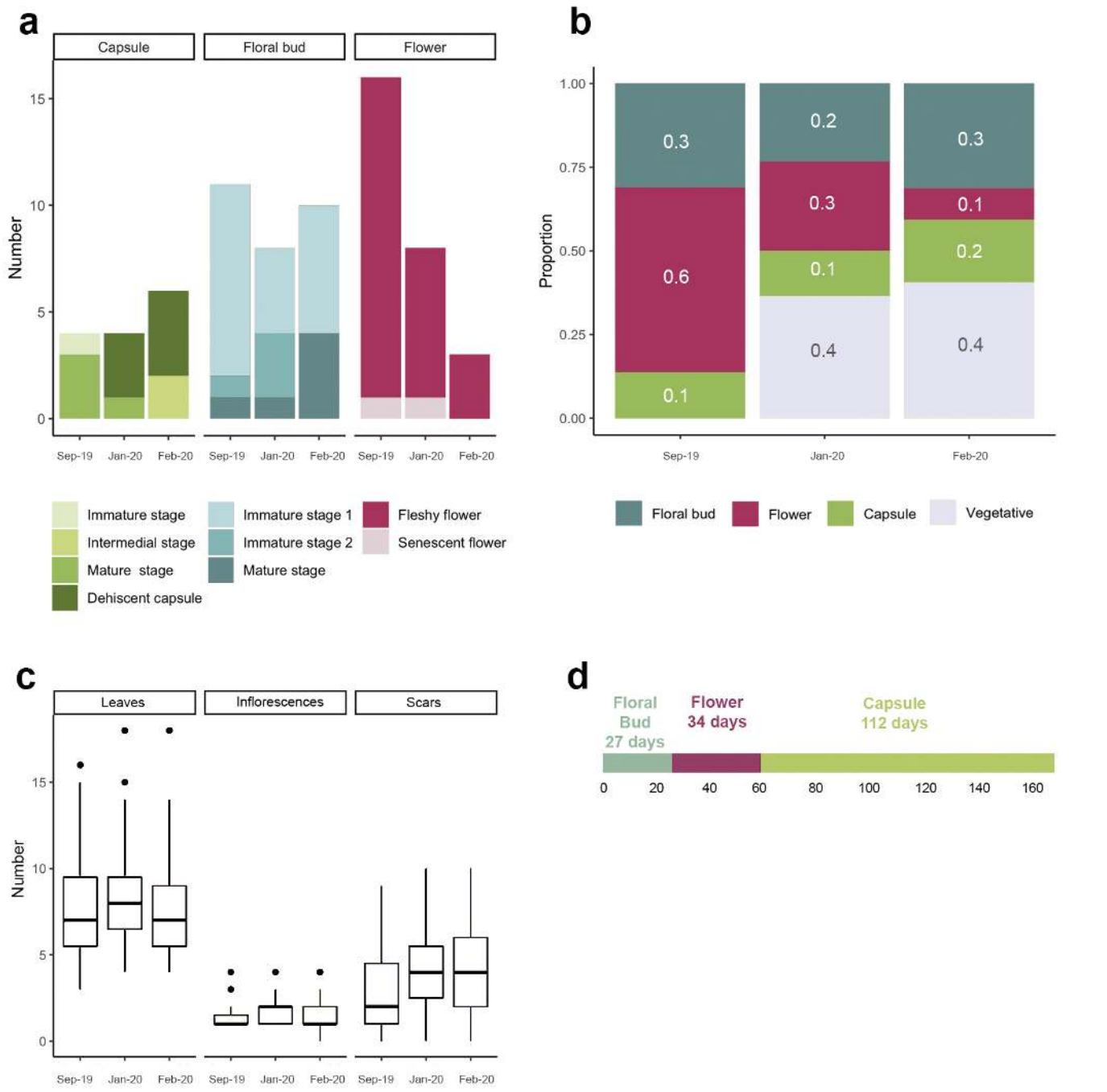

Figure 2

Phenological changes in population, plants, and flowers of *Masdevallia hortensis* during the sampling months. a. Number of plants with each reproductive stage. b. Proportion of phenological stages along sampling months (proportion labeled numbers are rounded). c. Distribution of number of leaves and inflorescences per plant and mean number of scars per inflorescence along sampling months. d. Estimated length of each floral phenological stage.

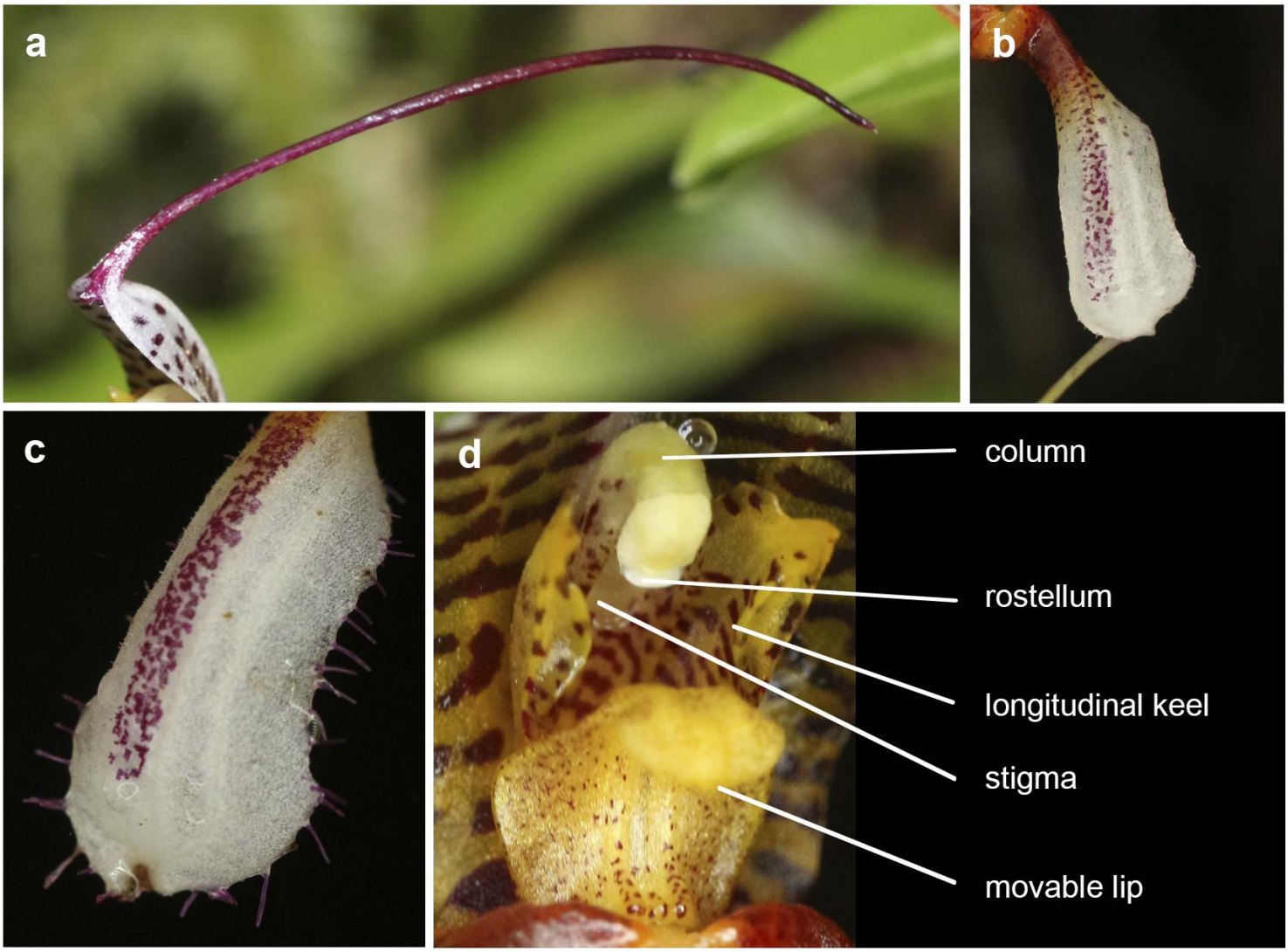

Figure 3

Key floral features related with visitors and pollinators behavior in *Masdevallia hortensis*: a. Sepaline tails; b. Adaxial lateral sepal surface. c. Single sepal, note the marginal trichomes. d. Inner whorl of perianth, androecium, and gynoecium. Photographs by Camilo Flórez-V.

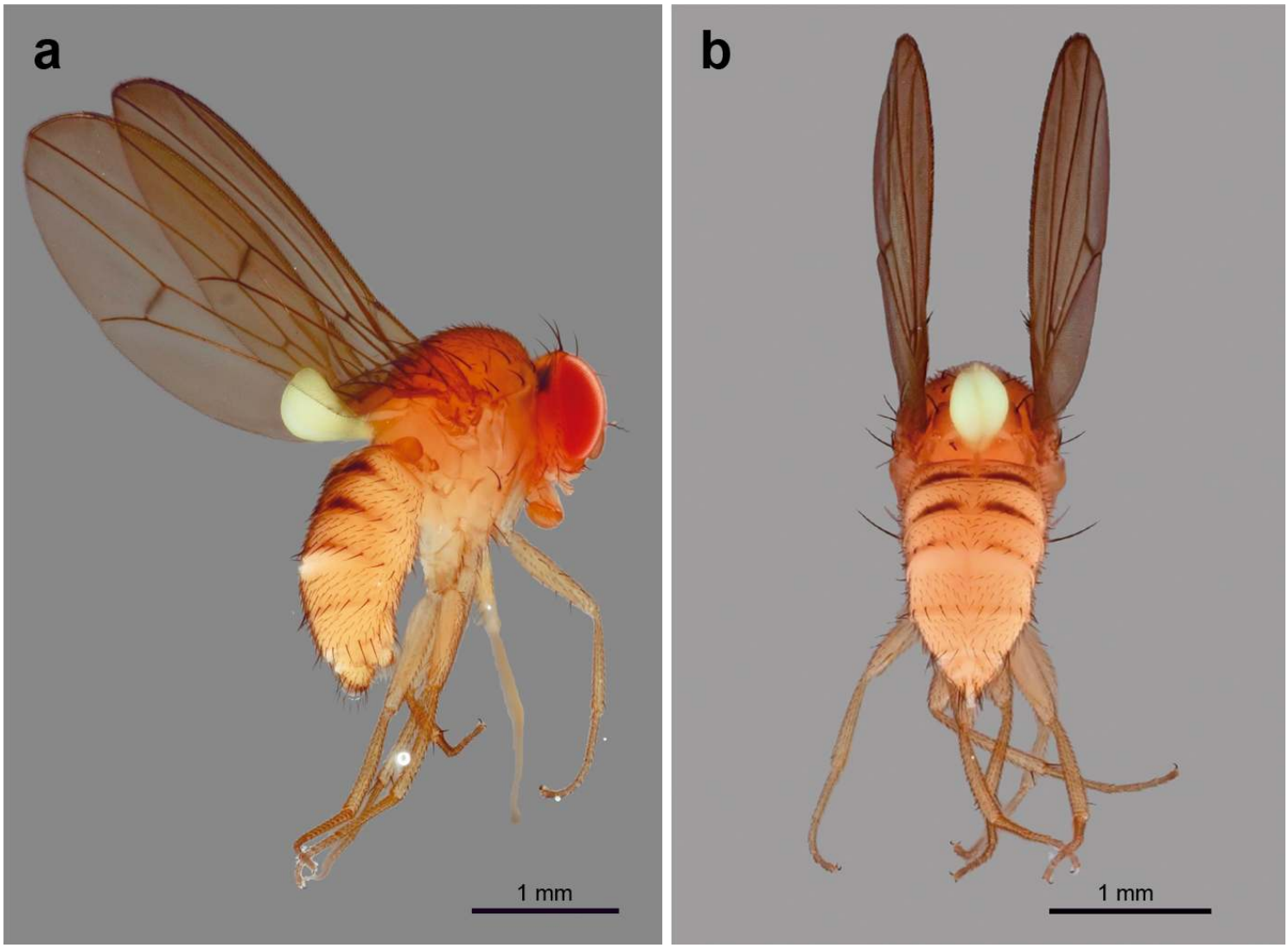

Figure 4

Pollinator of *Masdevallia hortensis* from the studied population, Drosophilidae sp. 1 [AO]: a. Lateral view with attached pollinarium, showing the attachment morphological position; b. Posterior view. Photographs by Camilo Flórez-V.

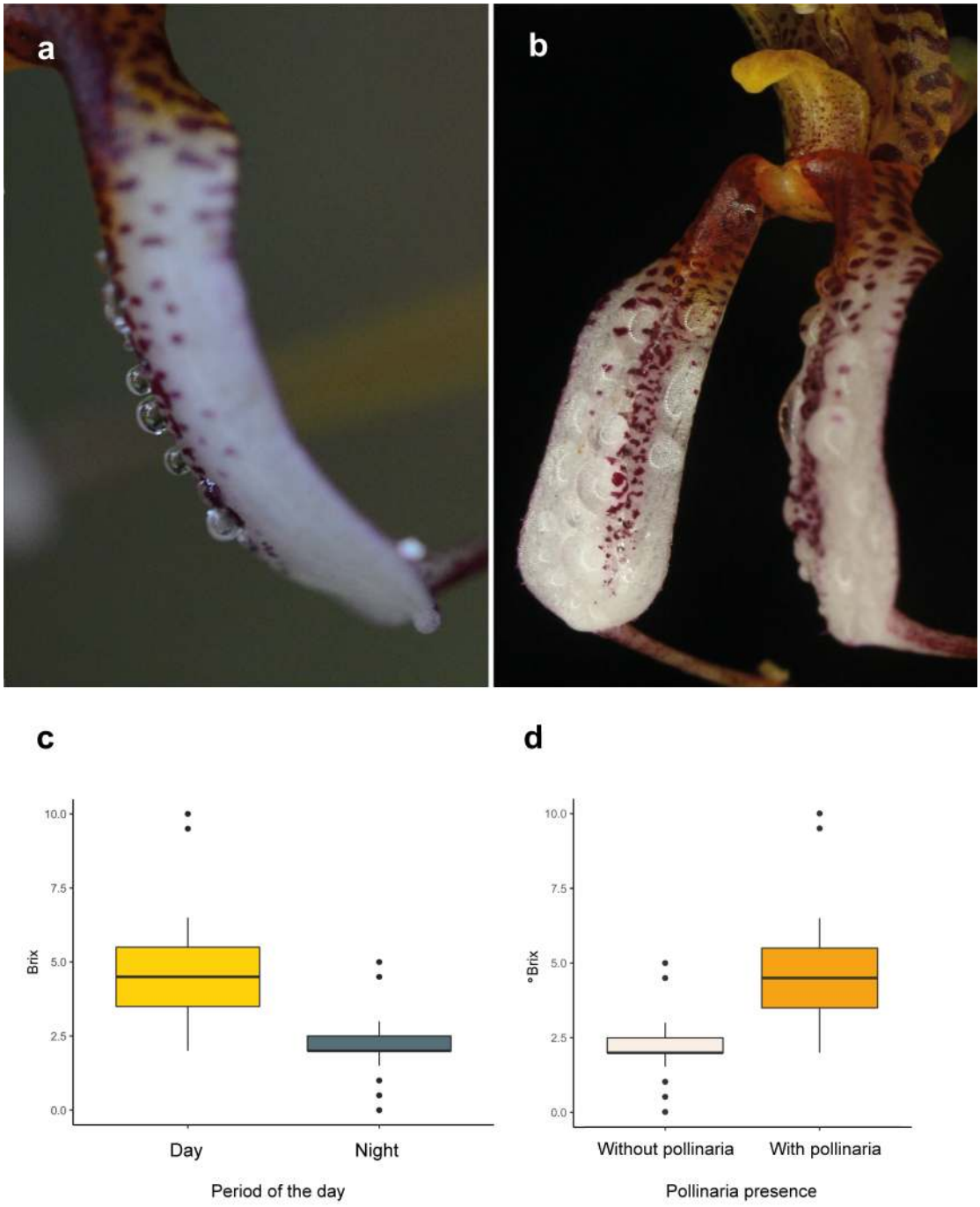

Figure 5

Secretions produced along dotted lines of the lateral sepals of *M. hortensis*; a. Discrete droplets. b. Gathered drops. c. Sucrose concentration in degrees Brix for flowers of *M. hortensis* at day and night. d. Sucrose concentration in degrees Brix for flowers of *M. hortensis* with and without pollinaria. Photographs by Camilo Flórez-V.

Figure 6

Pollination system in *Masdevallia hortensis*. a-c. Floral visitors sucking on substances from the sepals and lip surface. d. Drosophilidae sp. 1 [AO] trapped between the lip and column of *Masdevallia hortensis*. Photographs by Alejandro Hoyos and Camilo Flórez-V.

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