Pollinators of *Hoya pottsii*: Are the strongest the most effective?

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A R T I C L E   I N F O

A B S T R A C T

Hoya floral characters are highly elaborate and associated with a complex and specialised pollination mechanism. The pollination of two *Hoya* species has been studied previously, but little is known about the specific nature and interactions between flower morphology, pollinators, and their environment. Here we investigate the pollination of *Hoya pottsii*, where pollinaria are transferred onto several insects' legs and arolia including moths in the Erebidae family, ants, and a praying mantis. *Hypopyra vespertilio* (Erebidae, Erebinae) was the most effective at both carrying and depositing the pollinaria, *Colobopsis leonardii* (Formicidae) was shown to successfully insert only one pollinium whereas *Hymenopus coronatus* (Hymenopodidae) could only attach the pollinaria between its two euplantulae.

Several *Hoya* species were used to compare the effectiveness of pollinaria removal and insertion, pollinator size which was correlated to strength, floral scent, and morphology of the guide rail. The floral scent was dominated by Linalool, Methyl benzoate and Benzaldehyde which are known to attract moth, other species displayed similar scents but also showed many different compounds.

The effectiveness of a medium-sized moth in pollinating *H. pottsii* could be explained by the morphology of the guide rail which comprises a landing platform for the arolium. In *Hoya carnosa* the guide rail lacks a landing platform which could explain why stronger and larger moths were more effective in this species. The importance of the interaction between insect arolia and guide rails in the pollination of *Hoya* is illustrated and we suggest that their morphology corresponds with pollinator strength and how smoothly and precisely the pollinia can be inserted.

1. Introduction

Hoya, with around 300 species is the largest genus in Asclepiadoideae tribe Marsdenieae, in the family Apocynaceae. Distributed in SE Asia and Australia, with some species in India and Sri Lanka, most species are epiphytic, succulent vines developing extra-axillary flower clusters at the end of a thick persistent peduncle (Kleijn and Van Donkelaar, 2001; Wanntorp et al., 2014). Apocynaceae are mostly pollinated by insects (Hymenoptera, Diptera, Lepidoptera, Coleoptera, Hemiptera and Neuroptera), with few records of bird pollination (Ollerton and Liede, 1997; Ollerton et al., 2009, 2017; Pauw, 1998). The majority of the taxa that have been studied are pollinated by a single functional pollinator group (Ollerton et al., 2019).

Extreme synorganization of floral parts such as the androecium and gynoecium is characteristic of Apocynaceae (Endress 2016). These fusions may have led to the development of other features such as the

corona, pollinaria and guide rails, directing pollinators into a precise position within the corolla, and facilitating pollinaria removal and deposition (Endress 2016). *Hoya* corona lobes are folded and fused basally with the anther skirt, forming a sac-like structure where nectar secretion is located (Forster and Liddle, 1992; Kunze and Wanntorp, 2008). The primary nectary is located inside a slit on the anther skirt; its base forms a tube where the nectar accumulates and its apical part becomes cartilaginous on the sides and forms the guide rail. In most *Hoya* species the primary nectary in the slit has lost its nectar secretion function and is replaced by a secondary nectary formed by the corona lobes (Wanntorp and kunze, 2009). The pollinaria are formed from the thecae of adjacent stamens which become fused together, attached by the retinaculum and caudicle (translator arm) to the corpusculum (Fig. 1H). The corpusculum is a cartilaginous hollow structure with a channel in the centre that acts like a clip, attaching to the pollinator (Wanntorp, 2007). The pollinia have a thick margin on the inner side

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known as the pellucid margin, this margin gets trapped in the guide rail and pollen tubes will grow externally through the margin to reach the stigmatic surface. The guide rail morphology is of great importance in the pollination process but has received little attention in previous studies; it is here described in more detail (Fig. 1A).

The abovementioned floral characters, often associated with pollinator specialisation, have been considered critical to speciation and evolutionary radiation (Grant 1949; Stebbins 1970; Crepet 1983). However, synorganization evolution in some Apocynaceae taxa has also resulted in generalised plant-pollinator interactions (Waser et al., 1996).

Although morphological features of *Hoya* flowers are well understood, their function have only been studied in two species, *Hoya australis* (Forster, 1992) and *H. carnosa* (Mochizuki et al., 2017). The two studied species have different pollinators and the studies suggest that *Hoya* pollination is species specific. *Erebus esphesperis* (Eribideae), a nocturnal settling moth, was confirmed as the predominant pollinator in *H. carnosa* and capable of attaching the pollinaria on the aroliar pad. *Ocybadistes walkeri*, a diurnal skipper butterfly, was shown to be the main pollinator of *H. australis*, although ForsterFoster (1992) did not conduct experiments after dusk. Butterflies were capable of attaching the pollinaria on the legs and proboscis (although no exact region of the leg was mentioned).

Hoya species produce white flowers, strong nocturnal fragrance, and abundant nectar, which is strongly suggestive of moth pollination (Faegri and van der Pijl, 1979; Altenburger and Matile, 1988; Matile, 2006). Floral scents have been well studied in some asclepiad genera such as *Pachycarpus* (Shuttleworth and Johnson, 2012), *Xysmalobium* (Shuttleworth and Johnson, 2009), *Orbea* (Shuttleworth et al., 2017) and *Ceropegia* (Heiduk et al., 2016). Floral odour compounds emitted by flowers may provide an insight into the ecology and evolution of pollination systems (Dobson, 2006), but despite the large diversity of *Hoya*, studies on *Hoya* pollination and floral scent are scarce (Jürgens et al., 2010). In flowers of *Hoya carnosa* the scent is only produced at night (Altenburger and Matile, 1988), although this may differ in other *Hoya* species.

In this study, we investigated the pollinators of *H. pottsii* by capturing flower visitors during the evening and night. Pollinators were checked for pollinaria attachment as well as their location on the body and how many pollinia were missing, which is indicative of pollination effectiveness. Pollinator strength was estimated by using wing length as a proxy and this was correlated with their respective effectiveness. Guide rails were measured and observed by SEM and compared with *Hoya carnosa*. Further experiments with living pollinators were performed, hand pollination with severed moth legs and tools were explored to understand the fine details of such elaborate processes. Floral scent was extracted and compared with that of three other *Hoya* species including *H. carnosa, H. incrassata* and *H. heuschkeliana,* to find out if some compounds were specific to certain *Hoya* species.

2. Materials and methods

2.1. Pollinator observations

The research was conducted within the Xishuangbanna Tropical Botanical Garden in Southern Yunnan, China. *Hoya pottsii* is spontaneous and epiphytic, growing on several cultivated and subspontaneous trees in the garden. The species was selected because many fruits were produced every year, indicating an efficient pollination system (Fig. 2D). Foraging insects were observed during day and night but after several days' observations, we inferred an optimal pollination time to be between 2000 h and 2300 h, when pollinators were most abundant and seen landing on the flowers (Figs. 2A,B,F,G). The species flowered between the 13th and 30th of April in both 2018 and 2019 (17 days each year) and was observed for 102 h. Insect visitors were caught with a net and killed by crushing their abdomen or placing them in alcohol. The moths were identified using the volumes of Moths of Thailand (Pinratana, 1990) and iNaturalist (iNaturalist.org, 2019). Although almost all the larger moths and moths carrying pollinaria could be identified to species, many smaller moths could not be identified accurately (Supplementary data 1 and 2).
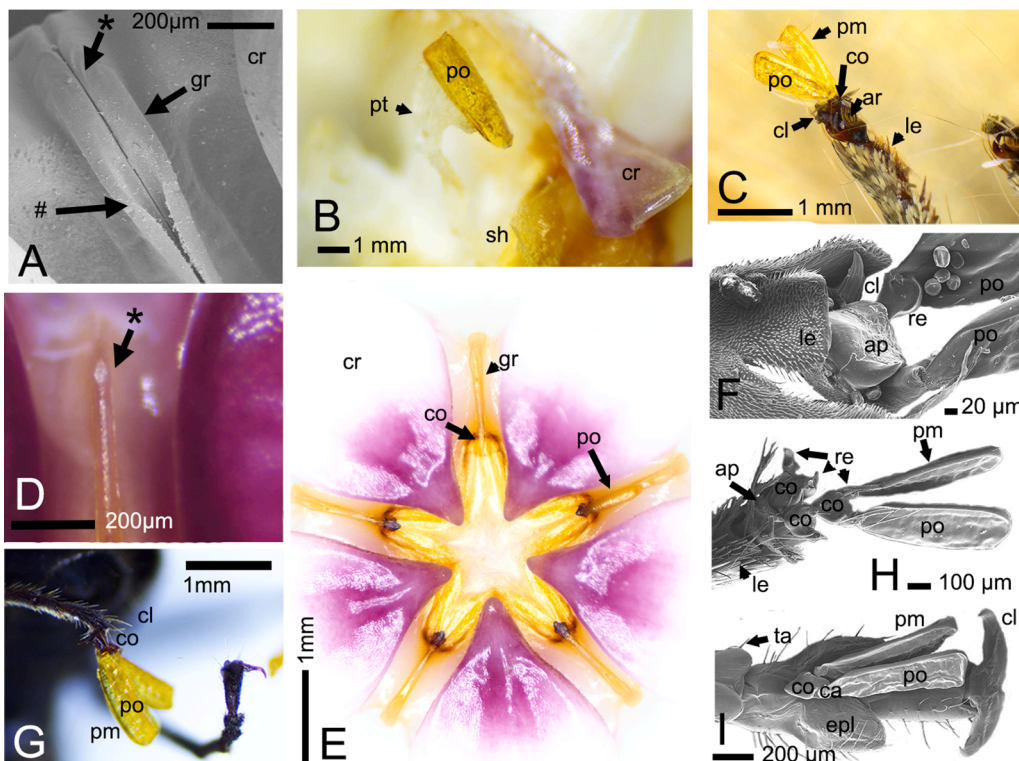
Fig. 1. Floral morphology of *Hoya pottsii* and pollinaria attachment on different pollinators. (A) Scanning Electronic Microscopy (SEM) detail of the guide rail with the narrowing part shown with # and the primary guide rail shown with *. (B) Corolla section after manual insertion of a pollinium followed by pollen tube germination through the pellucid margin. See arrow for pollen tubes. (C) *Hypopyra vespertilio* leg with attached pollinarium. (D) *Hoya carnosa* guide rail with insertion point shown with *. (E) Central part of the corolla with one pollinarium removed and one pollinium correctly inserted, see arrows po and co. (F) SEM of *H. vespertilio* leg with detail of corpuscula attachment on the arolium. (G) *Colobopsis leonardi* leg with attached pollinarium. (H) SEM of *H. vespertilio* leg with a single corpusculum and a chain of two corpuscula attached. (I) SEM of *Hymenopus coronatus* leg with attached pollinarium between the two euplantulae. ar, arolium; ap, aroliar pad; cl, claw; co, corpusculum; cr, staminal corona; epl, euplantulae; gr, guide rail; le, leg; pm, pellucid margin; po, pollinia; pt, pollen tubes; re, retinaculum; sh, stylar head; ta, tarsomere.

Fig. 2. Pollination of *Hoya pottsii*. (A) *Hypopyra vespertilio* flying towards an inflorescence. (B) *H. vespertilio* feeding on the nectar. (C) *Colobopsis leonardi* ant feeding on nectar. (D) Fruits forming after successful pollination. (E) *Hymenopus coronatus* or the orchid praying mantis waiting for its prey on top of an inflorescence. (F) *Diaphania indica* (Crambidae) or the cucumber moth feeding on nectar, but unsuccessful at removing pollinaria. (G) *Filodes fulvidorsalis* (Crambidae) feeding on nectar, this snout moth was not successful at removing pollinaria.

2.2. Flower morphology and measurements

Flower morphology and the pollination process were studied in detail using a stereomicroscope and scanning electron microscope (SEM, ZEISS/EVO LS10). Flower samples were dehydrated using an alcohol series, critical point dried, mounted on stubs with nail varnish and coated in platinum before observations. Flowers with successfully inserted pollinia were observed to check for pollen tube growth and we recorded the exact placement and orientation of the pollinaria. Pollen tubes were visible without preparation because the pollen tubes are grouped in a bundle and emerge from the pellucid margin (Mochizuki et al., 2017). Measurements of the guide rail were obtained using SEM and light microscope photographs.

2.3. Ant observations

Colobopsis leonardii ants were collected around the flowers in the early evening and placed inside pollination bags. We collected four bags with 15 ants in each and sealed the bags around four distinct inflorescences. We checked that no ants had pollinaria already attached to their legs and that no pollinaria had already been removed from the flowers. In two of the bags we put an inflorescence from a spontaneous plant that was growing more than 100 m away from the same site. The other two bags were put on inflorescences from potted plants in a polytunnel nursery, where no insects could enter. The bags were left overnight, ant legs and flowers missing pollinaria were then checked

and counted.

2.4. Pollinia removal and insertion

The corpusculum is a useful character in *Hoya* pollination because it stays attached to the pollinator, even after pollination (Wiemer et al., 2011). The ratio of the number of corpuscula and the number of remaining pollinia attached to pollinators allows us to get an estimate of the pollination effectiveness. This estimate is based on the fact that the caudicles are strong and do not detach from the pollinia unless cut with the sharp edges of the guide rail (Theiss et al., 2007). Similarly, the corpuscula are dark in colour and they are easy to observe, even without a microscope thus it is easy to check how many pollinaria have been removed from the flowers (Mochizuki et al., 2017).

2.5. Manual pollination

For hand pollination we used different techniques but all of them required the use of a stereomicroscope (Nikon C-PSN) that was transported on site. Inserting the pollinia, with their pellucid margin in the guide rail, required much trial and error. We used scalpels, tweezers, severed insect legs, hairs, steel wire, paper and glue. The easiest technique required the use of a 100 µm thick printing paper that was cut into a triangular shape with an acute tip. The tip of the triangle was applied along the guide rail and although the paper was too thick to be inserted, fibres from the edge of the paper would stick to the corpusculum,

attaching the pollinaria. These attached pollinaria would be dried for 15 min to allow for reconfiguration of the pollinia (Fig. 3E). The paper pieces would then be held with forceps and lightly deposited along the guide rail until one pollinium would slide within the rail and get cut from the caudicle. We also successfully used severed moth legs from *Hypopyra vespertilio* to attach and insert pollinia using a similar process.

2.6. Regression analysis

The forewing length of each pollinator was measured, and we used a regression analysis in IBM SPSS Statistics for Windows (Version 21.0), to fit a best curve between the forewing length and the number of pollinia

lost. The wing length could be a function of pollinator strength and the ability to successfully remove and insert pollinia (Mochizuki et al., 2017), and the number of pollinia lost is a function of pollination efficiency.

2.7. Scent collection and analysis

To collect floral scent, a dynamic headspace apparatus was applied to intact inflorescences. We used micro-vials filled with Tenax and Carbotrap (3–6 mm long), stuffed with a piece of glass wool on both sides of the adsorption material to keep it in place (following Dötterl et al., 2005). Inflorescences were enclosed in polyacetate (oven) bags for
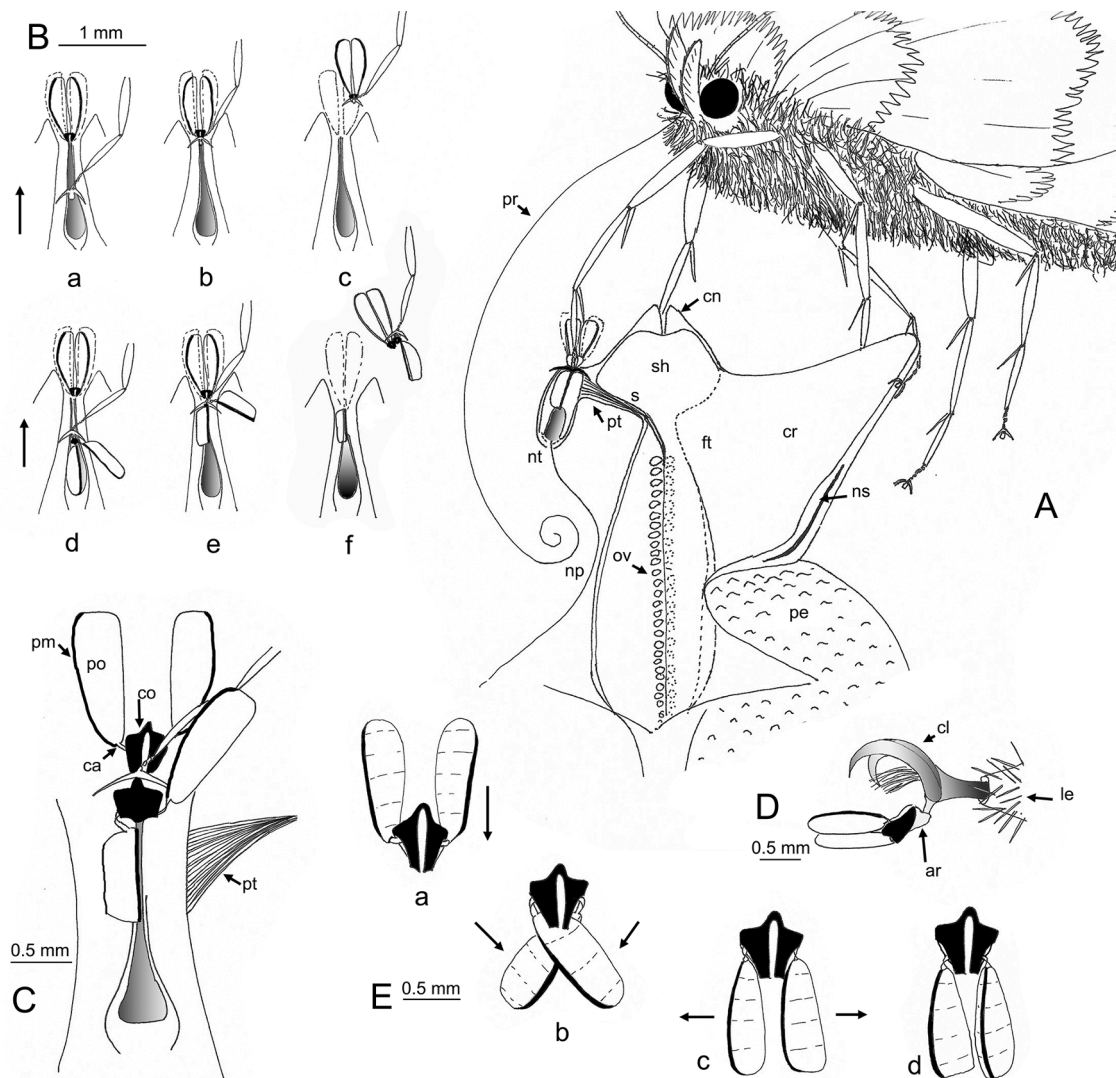
Fig. 3. Schematic representation of the pollinaria transfer and insertion in *Hoya pottsii*. (A) The moth reaches for the nectar that accumulates at the bottom of the anther skirt, by doing so its legs can get trapped in the guide rail (not to scale). (B) (a) A leg's claw and arolium is nested inside the insertion region of the guide rail. (b) The leg is pulled upwards and the arolium gets trapped by the narrowing guide rail. (c) The arolium moves upward from the guide rail to the corpusculum rail and gets trapped, the pollinarium is now securely attached to the arolium. (d) A leg with an attached pollinarium is nested inside the insertion region of the guide rail (the pellucid margin are facing downward). (e) The leg is pulled upwards and the arolium with the attached pollinarium gets trapped by the narrowing guide rail while the corpusculum stays on the surface, the caudicle is cut by the narrow edges of the guide rail and the pollinium pellucid margin is inserted in between the two rails where the pollen grains can germinate and reach the stigmatic surface. (f) The retinaculum moves upwards from the guide rail to the corpusculum channel and gets trapped, the pollinarium is now securely attached to the retinaculum of the previous corpusculum and a chain of corpuscula is forming. (C) Detail of (Bf) when the caudicle is cut and the retinaculum of the previous corpusculum gets trapped in a new corpusculum channel. (D) *Hypopyra vespertilio* aroliar pad with attached pollinarium. (E) Rotation of the pollinia after removal of the pollinarium. (a) the pollinia rotate 180° downwards. (b) The pollinia rotate inward and form a lock. (c) The pollinia rotate outwards. (d) the pellucid margin of both pollinia are now facing the same side as the corpusculum rail and are ready to be inserted in the guide rail. Ar, Arolium; Ca, Caudicle; Cl, Claw; Cn, connective appendage; Co, Corpusculum; Cr, Staminal corona; Ft, Filament tube; Gr, Guide rail ; Le, Leg; Np, nectary pool; Ns, secondary nectary; Nt, Nectary tube; Ov, Ovule; Pe, Petal; Pm, Pellucid margin; Po, Pollinia; Pt, Pollen tubes; S, stigmatic surface; Sh, stylar head.

volatile sampling. Scent-containing air was sucked through the micro-tube (flow rate 200 ml/min) with a battery-operated membrane pump (QC-1B, Beijing Ke An labour Insurance New Technology, Beijing, China). Samples were collected from 2000 h to 2130 h. The samples were analysed in the Central Laboratory of Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. The volatiles were analysed using an Agilent Technologies 7890A GC, equipped with an HP-5 MS capillary column (30 mm × 0.25 mm; film thickness, 0.25 mm) and a mass spectrometer 5975C (Agilent Technologies, USA) as detector. Helium was used as the carrier gas, at a flow rate of 1 ml/min. Injector and detector (MS transfer line) temperatures were both 250 °C. Column temperature was gradually increased from 40 °C to 100 °C at 3 °C/min, then ramped up to 250 °C at a rate of 20 °C/min and finally held for 10 min. Mass spectrometry was recorded at 70 eV with a mass range from m/z 29 to 540. Data were analysed using the program Chemstation (G1701EA E.02.02 MSD Productivity ChemStation Software, Agilent Technologies, Germany), and the NIST spectral database was implemented for preliminary identification of volatiles. Relative percentage amounts of the separated compounds were calculated automatically from peak areas of the Total Ion Chromatogram (TIC).

3. Results

3.1. Flower visitors

The flowers were visited soon after dusk, whereas visitor number diminished after 2300 h, until almost no more insects were seen after midnight. The flowers were visited by four orders of insect including ants (Hymenoptera), praying mantis (Mantodea), planthopper (Hemiptera) and moths plus butterflies (Lepidoptera). In total 420 individuals (not including ants) belonging to 17 families and 52 species were captured and seen feeding on the nectar during a total of 28 days of observations (Table 1). Most of the visiting moths that were seen feeding for a prolonged time belonged to the owlet moths and allied Noctuoidea superfamily. The underwings, tiger, tussock and allied moths (Erebidae) were the most common visitors with 76 individuals. One species of tussock moth (*Lymantria* sp.) was particularly abundant in 2018 but no individuals of this genus were captured in 2019, which could be due to a periodic life cycle, although most other species were observed equally during the two years.

The orchid praying mantis (*Hymenopus coronatus*) was not observed feeding on nectar but waiting for a prey on one inflorescence (Fig. 2E). Numerous ants were seen visiting the flowers to feed on nectar. They belonged to several species but only one species of exploding ants (*Colobopsis leonardi*) was found to carry pollinaria (Figs. 1G and 2C). Although the ants live in the canopy, they would reach ground level during the evening and even during the day. Even after flowering the ants would stay on the inflorescence axis and bite the fleshy axis, presumably for an unknown reward.

3.2. Insects carrying pollinaria

Out of the 420 individuals observed only 54 individuals carried pollinaria (Table 1). *Hypopyra vespertilio* was the most commonly captured moth carrying pollinaria, with 19 individuals in total and 100 pollinaria attached (11.1 pollinaria per moth on average). *Lygniodes hypoleuca* was also common with 9 individual moths collected but they only carried 21 pollinaria in total (2.3 pollinaria per moth on average). *Hypopyra vespertilio* had an average wing length of 36.9 ± 2.3 mm SD, *Lygniodes hypoleuca* average wing length was 44 ± 2.4 mm whereas the other larger moths carrying pollinia had an average wing length of 48 ± 6 mm. The largest moth carrying pollinaria was *Phyllodes consobrina* (62 mm wing length), with four pollinaria attached but no pollinia missing. Overall *Hypopyra vespertilio* inserted or lost 77 pollinia equalling to a rate of 0.36 ± 0.25. *Asota plaginota* (31.6 mm wing length) and *Cyana costifimbria* (24 mm wing length) were the only smaller moths that either

successfully inserted pollinia in the guide rails or lost pollinia. In total 182 pollinaria were removed from flowers and 95 pollinia were lost or inserted (proportion of missing pollinia of 0.26), (Table 1). The regression analysis showed that the best curve was the non-linear cubic regression with a coefficient of determination of 0.767 and $P<0.001$. A peak at around 28 mm forewing length can be observed when the number of pollinia lost is optimal (Supplementary data 2, Fig. 4). Several non-linear factors such as nectar feeding time, moths life span, pollinaria attachment availability, moths abundance as well as a minimum and maximum threshold when moths cannot remove pollinaria could not be included in this model (Fig. 4).

3.3. Pollinaria attachment

The moths were seen feeding on the nectar accumulated at the base of the anther skirt below the guide rail (Figs. 2B,F,G). Large moths struggled to get a hold on the slippery staminal corona and the reflexed petals, resulting in these moths often gripping the connective appendages and the sides of the staminal corona. All pollinaria were attached to the arolia and more specifically to its narrow edges and inner surfaces (Figs. 1C, F, H). Only in the orchid praying mantis the corpusculum was lodged in between the two euplantulae on the leg.

3.4. Pollinaria insertion

In *H. carnosa* the guide rail presents a small depression towards its base and when pressed with a 0.05 mm wide steel wire the rail will split open (Fig. 1D). The claws of most large moth are also around 0.05 mm at their base, but their ends are narrower; some strength may therefore be necessary to split the rail open. In *H. pottsii* the guide rail presents a wide area at its base to 0.114 mm wide narrowing to almost 0 mm (Fig. 1A), this area could act as a landing platform for the arolium (the arolium is around 0.1 mm wide). Upon landing the moth leg may slide upwards placing the arolium inside the wide part of the guide rail. When sliding up, the arolium may become clipped into the corpusculum and the pollinarium is removed (see Fig. 3 for detailed description). The insertion was successful with *H. pottsii* using pieces of paper with previously attached and reconfigured pollinaria but was not successful with *H. carnosa* because the paper may be too soft and did not have enough strength and rigidity to open the guide rail. We also noted that the paper technique did not allow formation of corpuscula chains which may require the arolium pressing down on the rail (See videos, supplementary data 3, 4, 5, 6).

Ants were ineffective pollinators because they did not survive overnight and died within a few hours. Nevertheless, within these few hours 25 ants out of 60 carried 48 pollinia and inserted one pollinium successfully (Table 1, Figs. 1G and 2C).

3.5. Scent

In the three flower samples, compounds representing three classes were detected (Table 2): monoterpenoids, benzenoid and phenylpropanoids. The scent was dominated by 2-phenylacetaldehyde (7.1%), methyl benzoate (27.4%), benzaldehyde (17.7%) and linalool (29.3%).

4. Discussion

In Asclepiadoideae the pollinaria attach mostly to insect mouthparts but 12 genera pollinated by Hymenoptera and five species pollinated by Diptera have pollinaria that attach to insect legs (Frost, 1965; Morse, 1981; Eisikowitch, 1986; Forster, 1992; Betz et al., 1994; Vieira and Sheperd, 1999; Ollerton et al., 2003; Johnson, 2005; Shuttleworth and Johnson 2008; 2009; Coombs et al., 2009; Fernandez et al., 2009; Wang et al., 2011; Nakahama et al., 2013; Cocucci et al., 2014; Mochizuki et al., 2017). *Hoya carnosa* and *Marsdenia megalantha* are the only Apocynaceae species that have been confirmed as having pollinaria

Table 1

Insect visitors of *Hoya pottsii* in Xishuangbanna Tropical Botanical Garden, as well as the number of pollinaria (corpuscula) attached during the observations in 2018 and 2019. In brackets are the number of pollinia left on the corpuscula. # were observed in captivity. **X** excluded data.

Order	Superfamily	Family	Subfamily	Species	No. of individual captured	No. of individuals carrying pollinaria	No. of pollinaria attached [No. of Pollinia left]	Proportion of pollinia missing	Average forewing length (mm)
Lepidoptera	Noctuoidea	Erebidae	Erebinae	*Erebus macrops*	3				67
				Phyllodes consobrina	1	1	4[8]	0	62
				Erebus caprimulgus	2	2	9[15]	0.17	48
				Erebus esphesperis	5	4	11[21]	0.04	47.3
				Lygniodes hypoleuca	8	8	21[38]	0.09	44
				Lygniodes sp.	1	1	2[4]	0	41
				Ericeia sp.	1				38
				Hypopyra vespertilio	20	16	96[110]	0.42	37.6
				Hypopyra sp.	3	3	5[8]	0.2	34.8
				Asota plaginota	16	4	15[27]	0.1	31.6
				spp.	15	1	1[2]	0	18
				Tinolius eburneigutta	1				27
			Arctiinae	*Barsine eccentropis*	1				10
				Barsine euprepioides	2				18
				Barsine sp.	3				18.5
				Creatonotos transiens	6				23
				Cyana costifimbria	8	3	6[10]	0.17	24
				Lyclene sp.	1				10
				Nyctemera adversata	1				26
				Spilosoma sp.	3				29.5
				Trischalis subaurana	1				8
			Lymantriinae	*Arctornis* sp.	32				18.4
				Cispia venosa	8	1	3[6]	0	33
				Lymantria sp.	26				30.9
				Lymantria sp. (pink)	1				34
		Nolidae		*Eligma narcissus*	2				35
		Notodontidae		*Gangarides vittipalpis*	1				43
				Tarsolepis remicauda	1				32
	Pyraloidea	Crambidae		*Bradina* sp.	18				18.6
				Glyphodes bivitralis	3				12.7
	Drepanoideae	Drepanidae		*Cyclidia substigmaria*	6				37.6
	Geometroidea	Geometridae		*Abraxas illuminata*	56	3	4[8]	0	28
				Amblychia angeronaria	1				45
				Comibaena sp.	2				18
				Thinopteryx crocopteres	4				29
		Uraniidae		*Acropteris iphiata*	1				24
				Micronia aculeata	14				23.9
	Papilionoidea	Nymphalidae		*Junonia atlites*	1				30
				Junonia lemonias	26	2	3[6]	0	29
				Parthenos sylvia	1				55
				Ypthima baldus	9				17

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Table 1 (*continued*)

| Order	Superfamily	Family	Subfamily	Species	No. of individual captured	No. of individuals carrying pollinaria	No. of pollinaria attached [No. of Pollinia left]	Proportion of pollinia missing	Average forewing length (mm)
		Pieridae		*Artogeia canidia*	20				25
				Delias descombesi	15	1	1[2]	0	40
				Eurema blanda	8				20
				Gandaca harina burmana	1				32
				Hebomoia glaucippe	1				45
	Pterophoroidea	Pterophoridae		spp.	1				16
	Bombycoidea	Saturniidae		*Cricula trifenestrata*	1				38
	?	?		spp.	52				15.9
Hemiptera	–	Flatidae		*Cerynia maria*	6				14
Mantodea	–	Hymenopodidae	*Hymenoponidae*	*Hymenopus coronatus*	1	1	2[4]	0	X
Hymenoptera	Formicoidea	Formicidae		*Colobopsis Leonardi* #	60	25	46[89]	0.03	X
					421	51	181[269]	0.26	30.1
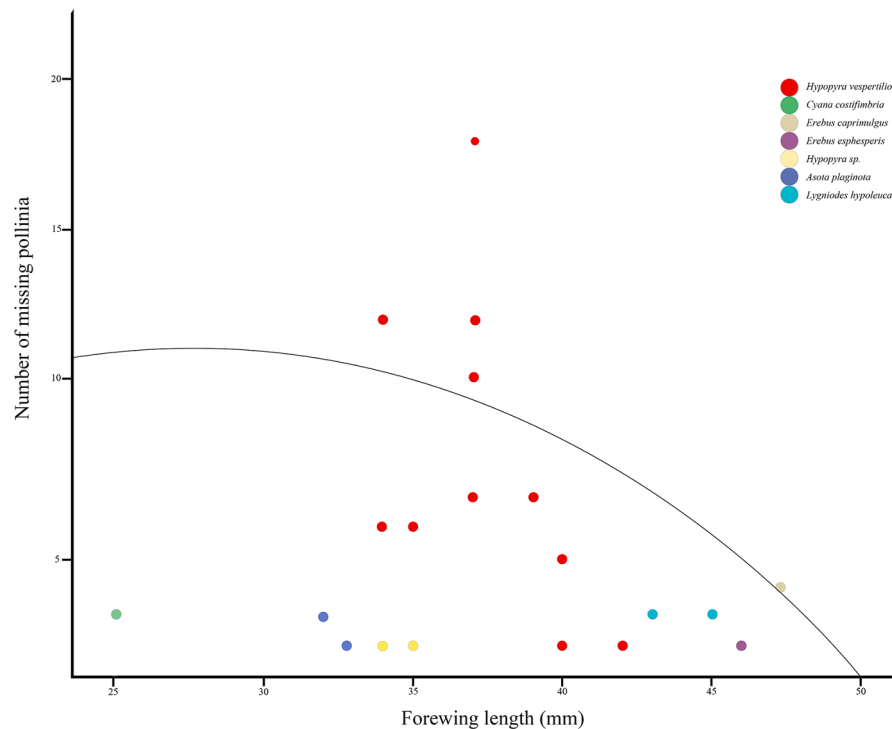
Fig. 4. Number of missing pollinia (pollination efficiency estimation) compared with the wing length (pollinator strength estimation) of captured pollinators. Non-linear cubic regression ($Y=-0.000228\times^3+0.572358X$), $R^2=0.767$ and $P = 0.00$ (Constant not included).

transfer on moth legs (Mochizuki et al., 2017; Domingos-Melo et al., 2019).

Forster (1992) and Mochizuki et al. (2017) suggest that the flower structure has adapted to these pollinators and that nectary location acted as a guide to the pollinarium attachment. It was suggested that the location of the primitive nectary in *H. australis* led to the attachment of the corpusculum to the proboscis as the nectary tube within the guide rail is still functional. In *H. carnosa*, as in most other *Hoya* species, the nectary function is replaced by secondary nectaries on the anther skirt and directed at the base of the corolla, therefore no insect would have their proboscis trapped in the guide rail. Instead, all pollinaria become attached to insect legs.

4.1. Correlation between pollinator size and pollinaria removal and insertion

In *Hoya pottsii* the predominant pollinator was identified as *Hypopyra vespertilo*, a nocturnal moth within the subfamily of predominantly large moths, the Erebinae (Figs. 2A, B; Supplementary data 1). With 100 pollinia removed and 77 pollinia missing (equalling to a rate of 0.28 ± 0.26) this moth carried and inserted or lost more pollinia than other moths, including *Phyllodes consobrina, Erebus esphesperis, E. macrops, E.*

Table 2

Floral volatiles in *Hoya pottsii* (H. pot) and the three species already published *H. heuschkeliana* (H. heu), *H.incrassata* (H. inc) and *H.carnosa* (H. car.) (Jurgens et al., 2010; Altenburger and Matile, 1998). Average relative amounts (in%) of floral scent compounds are listed according to compound class. tr = trace amounts. CAS # = CAS Registry Number. Volatiles that could not be identified were not included in the total of identified compounds.

	CAS#	**H. pot**	H. heu	H. inc.	H. car
Total number of compounds		**7**	13	19	6
Number of samples collected		**3**	2	1	?
Aliphatic compounds					
Alcohols					
Isoamyl alcohol	30,899–19–5	–	–	–	0–2.8
2-Nonen-1-ol	22,104–79–6	–	17.2	–	–
2-Decen-1-ol	22,104–80–9	–	14.9	–	–
Acids					
Nitrogen containing compounds					
Benzylnitrile	140–29–4	**6.5**	–	–	–
Benzenoids and phenylpropanoids					
Phenylacetaldehyde	100–51–6	**7**	–	0.1	–
Benzaldehyde	100–52–7	**17.6**	–	–	–
2-Phenylethyl alcohol	60–12–8	**7.8**	–	–	–
Methyl benzoate	93–58–3	**27.3**	–	–	–
Methyl salicylate	119–36–8	–	–	–	0–1.4
Monoterpenoids					
α-Thujene	2867–05–2	–	–	0.8	–
α-Pinene	80–56–8	–	0.4	1.2	Tr
β-Pinene	127–91–3	–	–	–	0–5.7
Camphene	79–92–5	–	–	1.5	–
Eucalyptol	470–82–6	–	–	–	0–11.4
Thuja-2,4(10)-diene	36,262–09–6	–	–	0.5	–
β-Phellandrene	555–10–2	–	–	7.7	–
Limonene	138–86–3	–	0.7	–	–
(Z)-Ocimene	3338–55–4	–	0.9	26.4	–
(E)-Ocimene	3779–61–1	–	37.5	–	–
γ-Terpinene	99–85–4	–	–	0.9	–
Unidentified monoterpenoid *m/z*: 81, 95, 137, 121, 136, 82, 93, 80, 109, 96		–	–	34	–
(Z)-Linalool oxide furanoide	5989–33–3	**3.7**	–	2.2	–
(E)-Linalool oxide furanoide	34,995–77–2	–	–	1.3	–
Linalool	78–70–6	**29.1**	20.9	–	0–85
(E,E)–2,6-Dimethyl-1,3,5,7-octatetraene	460–01–5	–	2.8	0.5	–
α-Terpineol	98–55–5	–	–	2.1	–
Unidentified monoterpenoid *m/z*:107, 135, 91, 151, 39, 150, 105, 109, 79, 122		–	–	7.4	–
Sesquiterpenoids					
β-Cedrene	546–28–1	–	–	0.3	–
(E)-Caryophyllene	87–44–5	–	0.6	–	–
(E)-Geranyl acetone	3796–70–1	–	2.0	–	–
(E,E)-α-Farnesene	502–61–4	–	0.1	–	–
Total percentage of identified compounds		**100**	98	45.5	100

caprimungulus and *Lygnioides hypoleuca* (Table 1). The rate was nevertheless much less than that observed in *H. australis* (success of 0.70, Forster, 1992) and *H. carnosa* (224 pollinia removed and 116 pollinia lost but only four pollinia inserted; a rate of 0.52 but success of only 0.03 if considering pollinia inserted, Mochizuki et al., 2017). *H. pottsii* was also visited by a wider variety of potential pollinators than the other two studied *Hoya* species, including seven moths that lost pollinia (*Phyllodes consobrina, Erebus esphesperis, E. macrops, E. caprimungulus, Lygnioides hypoleuca, Asota plaginota* and *Cyana costifimbria*, Supplementary data 1). Although *Erebus esphesperis* was recorded as the main pollinator of *H. carnosa* it was not very successful in *H. pottsii* (22 pollinia removed and 1 pollinium inserted, insertion rate of 0.04). Mochizuki et al. (2017) suggested that the pollination of *H. carnosa* required a moth that was strong enough to insert the arolium within the guide rail and therefore only the largest moth found in this area was able to succeed in this task.

However, this was not the case in *H. pottsii* and some larger moths such as *Erebus macrops* and *Phyllodes consobrina* were less successful, although some of the smaller moths such as *Asota plaginota* and *Cyana costifimbria* were also less successful. We therefore suggest that *Hoya pottsii* in Xishuangbanna is adapted to pollination by medium-sized moths and that the morphology of the pollinarium and guide rail can influence the selection of pollinators (Shuttleworth et al., 2017), (Fig. 4). Floral scent could also be associated with certain pollinators, although many volatiles are common between the species studied so far (Table 2), as well as with other moth pollinated flowers (Knudsen and Tollsten, 1993; Raguso and Pichersky, 1999; Jürgens et al., 2003; Dobson, 2006).

4.2. Ant-moth mixed pollination

Besides moths, one orchid praying mantis (Fig. 2E) and one ant species (Figs. 1G and 2C) were seen carrying pollinaria although only the ants were successful in inserting pollinia. Ants are common insects found in most ecosystems and known to feed on nectar including floral nectar (Peakall and Beattie, 1989). Pollination by ants has been recorded in 10 species in Asclepiadoideae (*Asclepias curassavica* (Chaturvedi and Pant, 1986), *A. exaltata* (Betz et al., 1994), *A. syriaca* (Kephart, 1979), *Ditassa rotundifolia* (Domingos-Melo et al., 2017), *Gomphocarpus physocarpus* (Coombs et al., 2009), and *Leptadenia reticulata* (Pant et al., 1982). Domingos-Melo et al. (2017) study on *Ditassa capillaris* and *D. hastata* pollination is the only verified case where pollinia have been observed to be successfully inserted by ants in the guide rail, followed by fruit production. Because ants died within a few hours in our experiment, we could not confirm their pollination effectiveness.

Chirango et al. (2019) recorded a mixed March fly, ant, and honeybee pollination system in *Eustegia minuta* (Apocynaceae, Asclepiadoideae). They concluded that March flies were the primary pollinators and that ants and honeybees only made a small contribution. In *Hoya pottsii* a mixed moth-ant pollination system is supported by our data, but the contribution from ants was not conclusive in our experiment. After flowering, ants were also observed to be staying on the young fruits and feeding on the inflorescence axis, potentially further protecting the development of seeds (Fig. 2D). Plants can attract ants by different morphological adaptations such as extrafloral nectaries, domatia, and food bodies (Rico-Gray and Oliveira, 2007; Rosumek et al., 2009). In return it is has been shown that ants will defend the host against herbivores and diminish fungal and microbial infections. However, in turn ants may also attack pollinators and reduce their pollination success (Villamil et al., 2018).

4.3. Corpuscula chains

With the exception of the orchid praying mantis, all pollinaria were attached on the insects' arolia, mostly on the margins but also on the central pad (Fig. 1C,F,H). This is a similar observation to Mochizuki et al. (2017) and confirms that attachment to other parts of the pollinator body is unsuitable, especially on hairs which would form a weak bond between the corpusculum and insect and do not allow for insertion. The arolium cuticle has microfolds in the form of grooves, running perpendicularly to the longitudinal axis of the pretarsus. Moreover, some secretion residues can be present on the inflated surface of the arolium (Beutel and Gorb, 2001). The arolium can therefore form a strong bond with the corpusculum which is essential during pollinia insertion. In this study we also recorded the attachment of several pollinaria and corpuscula in a chain (Fig. 1H). Each caudicle, when the pollinium is detached, can be inserted within the guide rail and then attach to another corpusculum. Although the link may become weaker after several corpuscula are attached, we observed up to seven corpuscula attached on one arolium, including one chain of four corpuscula and another of three. A similar observation was made in a study of the pollination of two South American species of *Morrenia* in Asclepiadoideae (Wiemer et al., 2011). The authors showed that these chains of

corpuscula are effective and can increase the pollination success of these species, although they suggest this can also hinder pollination when too many corpuscula get attached. Cocucci et al. (2014) studied corpusculum chains or concatenation and addressed why some species in the genus *Morrenia* had horns on the pollinaria preventing concatenation. No interference between the proximal and distal pollinia was found in the species where pollinaria attached to the legs, but they suggested that in other species the horn evolved in response to male-male competition between pollinaria. Nevertheless, to form a chain with another pollinarium, one pollinium needs to be inserted. It is not clear how stopping concatenation can increase pollinarium success, except if several pollinia need to be inserted in a flower for efficient pollination or if the previous pollinium can be displaced. The chain of corpuscula has not been observed in other species of *Hoya* and may be associated with the morphology of the corpusculum, especially the size of the retinaculum, caudicle and corpusculum.

4.4. Is the guide rail morphology adapted to certain moths?

Several breeders have noted that upon removal the pollinia move inwards and cross over forming a clasp. This clasp was suggested as a way that the pollinarium can get trapped by the insect leg, but this would not be strong enough during the insertion. Instead we observed that every corpusculum was attached to the arolium by a channel in the corpusculum which upon sliding up gets caught on the arolium or its edges. One important feature in the removal and insertion of the pollinarium is the morphology of the guide rail (Fig. 1A,D). This was never described before but differs significantly between species such as *H. carnosa* and *H. pottsii* and could explain why medium sized moths can be efficient pollinators. In *Hoya pottsii* the lower part of the guide rail is wider and not hollow forming a landing platform for the arolium and pollinia. When the moth's leg slides upwards into the guide rail the arolium and pollinia get trapped without too much strength. Although we have not observed the process in *H. carnosa*, we suggest that the same process would require more strength because the claw would first need to split open the guide rail.

Difficulties in designing tools and methods to manually pollinate the flowers have limited the scope of this research. We hope that in the future we can test this more rigorously with a larger sample size when manual pollination becomes easier.

4.5. Volatile compounds in Hoya are diverse and could attract specific moths

Asclepiadoideae shows a wide variety of volatile compounds, representing 13.2% (237 compounds) of the total floral scent compounds gathered from 991 seed plants species (Knudsen et al., 2006). Only three species of *Hoya* have been previously investigated for their floral scents; *Hoya carnosa* (Altenburger and Matile, 1988; Matile and Altenburger, 1988; Kaiser, 1994), *H. incrassata* and *H. heuschkeliana* (Jürgens et al., 2010). Only the pollinators of *H. carnosa* in Japan are known, so we could not compare with the other two species. *H. carnosa* produced six compounds including linalool, isoamyl alcohol, beta-pinene, eucalyptol and methyl salicylate. Only linalool was common between *H. carnosa* and *H. pottsii* and it was also the most abundant compound in *H. pottsii*. Both of the main compounds, methyl benzoate and linalool found in *H. pottsii*, are commonly reported in the floral odours of moth-adapted plant species (Knudsen and Tollsten, 1993; Raguso and Pichersky, 1999; Jürgens et al., 2003; Dobson, 2006). The attractiveness of methyl benzoate and linalool for noctuid moths has been demonstrated in upwind flight experiments in wind-tunnel systems (Plepys et al., 2002; Dötterl et al., 2006). Methyl benzoate was not found to occur in *Hoya incrassata* and *H. heuschkeliana* but linalool was present in *H. heuschkeliana* (Jürgens et al., 2010). Both *Hoya* species had high relative amounts of monoterpenoids, but while the scent of *H. incrassata* was dominated by (Z)-ocimene and an unidentified monoterpenoid, the

scent of *H. heuschkeliana* was dominated by (E)-ocimene in combination with linalool (Jürgens et al., 2010).

Based on difference in scent profiles, we can speculate that the difference in pollinators between *H. carnosa* and *H. pottsii* may be a result of the volatile compounds produced, although electrophysiological analyses would be necessary to test this hypothesis further. Our results also show that the guide rail has adapted to certain moths by facilitating the insertion of the pollinia. Pollinator behaviour, including feeding habits, scent attraction, travelling distances and how legs and arolia function are still unknown, thus limiting our understanding of *Hoya* pollination.

Author contribution

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.flora.2020.151734.

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