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RESEARCH PAPER

Pollination ecology of the Neotropical gesneriad *Gloxinia perennis*: chemical composition and temporal fluctuation of floral perfume

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ABSTRACT

- Although common among orchids, pollination by perfume-gathering male euglossine bees is quite rare in other Neotropical families. In *Gesneriaceae*, for example, it is reported in two genera only, *Drymonia* and *Gloxinia*. Flowers of *G. perennis* are known to emit perfume, thereby attracting male euglossine bees as pollinators. However, detailed reports on the pollination ecology, as well as on chemistry of floral perfume of individuals in natural populations, are still missing. In this study, we report on the pollination ecology of *G. perennis*, focusing on the ecological significance of its floral perfume.
- In natural populations in Peru, we documented the floral biology and breeding system of *G. perennis*, as well as its interaction with flower visitors. We also characterised the chemical composition of floral perfume, as well as its timing of emission.
- *Gloxinia perennis* is self-compatible and natural pollination success is high. Spontaneous self-pollination occurs as a 'just in case strategy' when pollinators are scarce. Perfume-collecting males of *Eulaema cingulata* and *El. meriana* were identified as pollinators. The perfume bouquet of *G. perennis* consists of 16 compounds. (*E*)-Carvone epoxide (41%) and limonene (23%) are the major constituents. Perfume emission is higher at 09:00 h, matching the activity peak of *Eulaema* pollinators.
- Flowers of *G. perennis* have evolved a mixed strategy to ensure pollination (*i.e.* self- and cross-pollination), but cross-pollination is favoured. The size and behaviour of *Eulaema* males enables only these bees to successfully cross-pollinate *G. perennis.* Furthermore, *G. perennis* floral perfume traits (*i.e.* chemistry and timing of emission) have evolved to optimise the attraction of these bees.

INTRODUCTION

In Neotropical forests, flowers of hundreds of plant species produce perfume instead of nectar or pollen as reward for pollinators. The perfumes, mainly terpenes and aromatics, are exploited only by males of euglossine bees (Apidae: Euglossini), which inadvertently pollinate flowers while collecting them. The collected perfume in flowers is stored in specialised leg pockets and exposed in perching sites during courtship display, most probably to attract con-specific females (Eltz *et al.* 2003; Zimmermann *et al.* 2006).

Pollination by perfume-collecting euglossine males occurs in about 850 species (Ramírez 2009). This pollination syndrome is far more common among orchids – thus the colloquial name 'orchid bees' – but is also reported in many other families, such as *Araceae*, *Arecaceae*, *Euphorbiaceae*, *Solanaceae* and *Gesneriaceae* (Dressler 1982; Roubik & Hanson 2004). Among *Gesneriaceae*, pollination by perfume-collecting euglossine males is confirmed in only two genera, *i.e. Gloxinia* L'Hér. and *Drymonia* Mart. (Vogel 1966; Dressler 1968, 1982; Roubik & Hanson 2004). Other Neotropical gesneriads, such as those belonging to the genus *Monopyle* Moritz ex Benth. (Wiehler 1983; Vogel *et al.* 2005), are also believed to be pollinated by male euglossine bees, but this assumption is based only on floral characters (syndrome). However, in timed observations at a flowering *Monopyle* sp. population in south Ecuador, no pollinator was observed (G. Gerlach, personal observation).

Gloxinia perennis (L.) Druce is one of the most emblematic *Gesneriaceae*, mainly because of its unusual floral characteristics. It was first described by Linnaeus (1753) as *Martynia perennis* L. (*Martyniaceae*) and then designated as the type species of *Gloxinia* (as *Gloxinia maculata* L'Hér.; Aiton 1789). It presents large, strongly scented violet flowers, which do not offer pollen or nectar as reward for pollinators (Vogel 1966). Pollination observations in *G. perennis* date back to Crüger's work (1864), who noted "its flowers are visited by the same insects as *Catasetum*" (*i.e. Eulaema* bees). However, the exact motivation of *Eulaema* bees to visit flowers of *G. perennis* remained unclear until the seminal work of Stefan Vogel (1966) – "Parfümsammelnde Bienen als Bestäuber von

Orchidaceen und *Gloxinia*." In his work, Vogel comprehensively described the perfume-collecting behaviour of *Eulaema* males (and typical of many other euglossine bees) at 'perfume flowers' of *G. perennis* and many orchids.

Flowers of *G. perennis* emit a strong caraway-like fragrance which is typical to many *Catasetum* species (*e.g. C. expansum* Rchb.f., *C. macroglossum* Rchb.f., *C. longifolium* Lindl and *C. saccatum* Lindl.) that are pollinated by *Eulaema* species (Gerlach & Schill 1991; Milet-Pinheiro & Gerlach 2017). Floral scents are known to be directly involved in the selective attraction of euglossine males by perfume-rewarding orchids (Hills *et al.* 1972; Williams & Whitten 1983; Milet-Pinheiro & Gerlach 2017), and this might also be true for *G. perennis*. Indeed, field observations point to a selective attraction of *Eulaema* males by flowers of *G. perennis*; Vogel (1966) reported visits by males of *El. nigrita* in Brazil, Dressler (1968) by *El. meriana* in Panamá, Vogel *et al.* (2005) also by *El. meriana* in Costa Rica and Witschnig *et al.* (2008) by *El. meriana* and two sporadic species of *Euglossa* also in Costa Rica. In the aforementioned works, species of *Eulaema* were considered the only pollinators of *G. perennis*, but it should be mentioned that these observations were performed in either cultivated or naturalised populations.

In perfume-rewarding plants, it is largely assumed that perfume emission is higher in the morning than in the afternoon (Hills *et al.* 1972; Dodson 1978; Hills 1989; Hills & Williams 1990), possibly as a response to the time of higher pollinator activity (Armbruster & McCormick 1990). Curiously, this long-held assumption was historically based on human olfaction and has only recently received experimental support. Using chemical analytical methods for quantification, Milet-Pinheiro *et al.* (2015) found that the peak of perfume emission in flowers of *C. uncatum* Rolfe matches the peak of *Euglossa* activity on flowers (09:00–12:00 h), and suggested that this daily fluctuation would be a strategy to save the high energetic cost of perfume production when activity of pollinators is normally low or absent. If this was true, the patterns of perfume fluctuation in species pollinated by *Eulaema* should be somewhat different. In nature, activity of *Eulaema* bees on perfume-rewarding flowers was recorded as higher between 06:00 and 09:00 h (Dodson 1978; Janzen 1981; Carvalho & Machado 2002; Witschnig *et al.* 2008). Thus, the peak of perfume emission in species pollinated by *Eulaema* should be earlier than in species pollinated by *Euglossa* (Milet-Pinheiro & Gerlach 2017).

In the present study, we aimed to investigate the pollination and reproductive biology of *G. perennis* in natural populations in the Peruvian Andes and lowlands, emphasising the chemical composition and daily fluctuations in its floral perfume. Assuming that pollinators of *G. perennis* in naturalised populations are the same or closely related to those in native populations, we speculate that chemistry and daily fluctuations of floral perfume in *Gloxinia perennis* will be adapted to *Eulaema* bees.

MATERIAL AND METHODS

Study site

Field studies were carried out during the flowering seasons in two locations of the rain forest of central Peru: (i) Pozuzo (10.068540° S, 75.551173° W) at 740 m a.s.l. (February 2015) and (ii) Panguana Private Reserve (9.613459° S, 74.934257° W) at 230 m a.s.l. (March 2016). The studied population in Pozuzo is in the local cemetery and riverbanks of the Huancabamba River, and the population of Panguana on riverbanks of the Yuyapichis River. Both localities have similar weather conditions but mean annual temperature and precipitation are higher in Panguana (26 °C and around 3,000 mm of rain; Schönitzer & Feuerabendt 2014) than in Pozuzo (22.6 °C and around 2,000 mm of rain; Espinoza Villar *et al.* 2010). Fieldwork was carried out with permission from the Dirección General Forestal y de Fauna Silvestre (Peruvian Ministry of Agriculture and Irrigation; Resolución de Dirección General N° 0096-2015-SERFOR-DGGSPFFS).

Plant species

Gloxinia perennis is a rhizomatous herb (Fig. 1A), which is widely distributed across the Andes, from Venezuela to Bolivia, and is probably naturalised outside the Andes and Amazonia (*e.g.* Central America and the Guianas; Boggan *et al.* 1997; Skog 1979). Plants of *G. perennis* bear campanulate violet flowers (Fig. 1B) with an inner dark violet osmophore and no nectary (Vogel *et al.* 2005). They grow in sunlight-exposed areas, frequently next to trails, roads and riverbanks. In populations from Peru, the blooming period is from January to April (C. Martel, personal observation). Vouchers of *G. perennis* were deposited at the Herbarium of Universidad Nacional Mayor de San Marcos (USM) in Lima, Peru, and Botanische

Fig. 1. *Gloxinia perennis*: (A) a dense patch and its habit, and (B) details of one inflorescence with two flowers. Photographs by Paulo Milet-Pinheiro.

Staatssammlung München (M), Germany, under number GG-923.

Flower morphometry

We calculated means and coefficients of variation (CV; *i.e.* ratio of SD to mean, expressed as a percentage) for several morphological traits: (i) corolla length, (ii) height and (iii) width at the corolla entrance, (iv) height and (v) width at the corolla base, (vi) stamen length and (vii) style – including the stigma – length (Fig. 2). The CV indicates the extent of variability in relation to the mean and is calculated as the ratio of the SD to the mean; it allows evaluation of the relative variability of the different floral characters. Measurements were taken from 32 flowers in different individuals (n = 1 per plant) using a caliper. Stamen and pistil length were recorded on different days after flower opening because protandry has been reported in *G. perennis* (Vogel 1966).

Flower anthesis and pollination success

To investigate flower anthesis (*i.e.* the period during which the flower is fully open), 21 flower buds of different individuals (n = 1 per individual) were marked with flagging tape and covered with net bags (8 × 3.5 cm) 1 day before opening, to avoid interference of pollinators during anthesis. The flowers were then observed daily from opening until abscission. We recorded flower longevity and the time of flower opening, anther dehiscence and stigma receptivity. Flower anthesis was followed in plants from both Panguana and Pozuzo. Stigma receptivity was determined with H_2O_2 solution (10%; Dafni *et al.* 2005) in Pozuzo and using Peroxtesmo KO indicator paper test (Macherey-Nagel, Düren, Germany) (Dafni & Maués 1998) in developed buds and open flowers from Panguana.

Pollination success and breeding system

The next section describes only measurements carried out in Panguana, since most of our samples from Pozuzo were lost due to unexpected events (*i.e.* most plants of the sampled population were damaged by human actions). To determine the breeding system of *G. perennis*, pre-anthesis buds were covered with voile bags and submitted to the following procedures at the start of anthesis: (i) spontaneous self-pollination – flowers were kept bagged without manipulation; (ii) autogamy – flowers were hand-pollinated with self-pollen; (iii) geitonogamy – flowers were hand-pollinated with pollen from different flowers of the same plant; and (iv) xenogamy – flowers were hand-pollinated with different pollen donors from plants 100 m distant. Furthermore, (v) control flowers – open flowers were labelled and freely exposed to pollinators. For each treatment, we used 16 flowers. In total, 52 individuals were used for the breeding system experiment and the number of flowers used per individual ranged from one to two. For hand-pollination, we removed anthers from flowers using forceps and used these to deposit pollen on the stigmatic surface. After manipulation, flowers were bagged again and bags were only removed after corolla abscission. One month after the start of experiments, fruits were recorded and collected to count seed number. For each treatment and for the control, we measured seed set as the mean number of seeds per fruit. Fruit set was not established among treatments because some of the marked flowers could not be found (see Results). In addition to the breeding system experiments, we estimated the natural pollination success by marking 262 newly opened flowers in Panguana, which were left uncovered to allow free visitation by animals. We then recorded the number of fruits and estimated natural pollination success as the number of fruits set in relation to the total number of marked flowers.

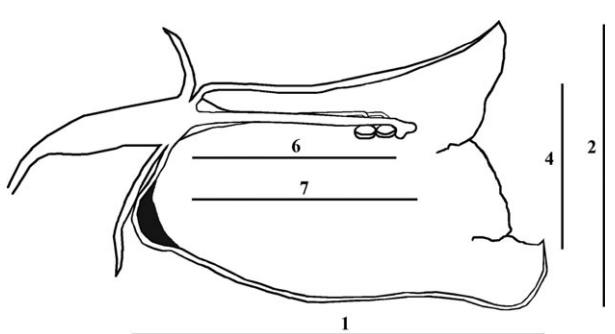

Fig. 2. Morphometric variables studied on flowers of *Gloxinia perennis*: (1) corolla length; (2) corolla height at the entrance; (4) corolla height at the base; (6) stamen length; (7) style length. The other two analysed morphometric variables (3) width of corolla entrance and (5) width at the corolla base (see text) are not shown. Note the osmophore at the base in black colour.

Flower visitors and effective pollinators

The frequency of visitors was recorded on flowers of 24 individuals in Pozuzo and 72 individuals in Panguana for 4 and 12 days, respectively. In Pozuzo and Panguana, floral visits to two groups of three flowers belonging to different plants (n = 1 flower per individual) were recorded at intervals of 15 min per hour from 06:00 to 16:00 h each day using a video camera (Panasonic HC-X810). Flower visits were also documented using individual photographs (Canon Powershot G16 and Canon EOS 400D). Based on direct observations and on the recordings, we determined the resource sought by each visitor species and describe their behaviour on flowers. Effective pollinators were determined based on contacts with stigmas and anthers, flights performed among conspecific plants and frequency of visits. Additional direct observations on flowers were performed on one night (from 20:30 to 05:30 h) to check for activity of nocturnal animals.

Sampling of floral volatiles

Using dynamic headspace methods, floral perfume samples of *G. perennis* were collected *in situ* (Pozuzo) for two purposes: (i) to chemically characterise its perfume bouquet, and (ii) to measure possible daily fluctuations in perfume emission. Intact flowers from ten individuals (n = 1 flower per individual) were individually enclosed in a polyester oven bag (6 × 3 cm; Toppits®, Germany) for 5 min, after which the volatiles were trapped for 3 min in a tube containing adsorbent using a membrane pump (Rietschle Thomas, Puchheim, Germany) at a constant flow rate of 200 ml·min^{-1}. The tubes with adsorbent consisted of Chromatoprobe quartz glass microvials (Agilent, Santa Clara, CA, USA; length: 15 mm; inner diameter:

2.5 mm) filled with 3 mg of a 1:1 mixture of Tenax™ TA (60/80 mesh; Supelco, Bellefonte, PA, USA) and Carbotrap B (20/40 mesh; Supelco). For each flower, four samples each at different time intervals were collected: (i) 06:30–07:00 h, (ii) 09:30–10:00 h, (iii) 12:30–13:00 h and (iv) 15:30–16:00 h. Before perfume collection, flowers were labelled and covered with voile bags. Samples were taken from flowers on their first day of anthesis. We also collected perfume samples from Panguana, but as the sample size was small per each time interval (*i.e.* n = 3), we did not include these in our analysis. To detect environmental contaminants, negative controls (empty bags; n = 5) were collected in parallel using the same aforementioned protocol. Adsorbent tubes were stored in 2 ml screw cap vials at −20 °C until chemical analyses (see below).

Chemical analysis of floral perfume

To identify the volatiles in the floral perfume of *G. perennis* and quantify daily fluctuations in scent emission, headspace samples were analysed on a coupled gas chromatograph–mass spectrometer system (7890B GC - 5977A MSD; Agilent Technologies, Waldbronn, Germany), equipped with a thermal desorption unit (TDU; Gerstel, Mülheim an der Ruhr, Germany) and a cold injection system (CIS 4C; Gerstel). The GC was equipped with a DB-5MS capillary column (30 m × 0.25 mm i.d. × 0.25 µm film thickness; J&W Scientific, Folsom, CA, USA). Helium was used as carrier gas with a constant linear velocity of 1 ml·min^{-1}. Chromatoprobes were thermally desorbed at 300 °C for 10 min and refocused with liquid nitrogen. Oven temperature started at 50 °C for 1 min, then raised to 310 °C at a rate of 4 °C·min^{-1}. The mass spectrometer was run in electro-ionisation (EI) mode (70 eV) and set to a scan range from 35 to 450 mz^{-1}, with a scanning rate of 3.5 scans s^{-1}. Data were processed and analysed on the MSD Chemstation Software (Agilent Technologies, Palo Alto, CA, USA).

Compounds were identified by comparing their mass spectra and retention indices with those of authentic reference samples available from commercial mass spectral libraries (NIST11, Wiley Registry™ 9th Edition and ADAMS) and the library of the Institute of Evolutionary Ecology and Conservation Genomics, Ulm University, Germany, integrated to the software Agilent MSD Productivity ChemStation (Agilent Technologies). Confirmation was obtained by comparison of retention times with published data and authentic standards. The peak areas on the chromatograms were integrated to obtain the total ion current signal, which was used to determine the relative percentage of each compound. The daily fluctuation in scent emission was calculated as the mean relative amount of the total scent emitted at each time interval in relation to the scent emitted in all time intervals. Toluene (1 µg) was injected directly into the adsorbents tubes and used as internal standard.

Statistical analyses

Floral traits associated with specific pollinator guilds are often correlated as they might be the result of selection pressure exerted by pollinators. This study, unlike previous studies, did not solely focus on chemical traits, but also investigated whether flower morphometric traits were correlated. Therefore, Pearson correlation test and principal components analysis were used. Floral morphometric values were natural log-transformed prior to statistical analysis to eliminate heteroscedasticity. Style and stamen length were excluded from the analyses as these change (elongate and retract) during floral ontogeny (see Results).

To assess whether seed set varied among treatments, we used a non-parametric Kruskal-Wallis test. Mann-Whitney *U*-tests, followed by a Benjamini-Hochberg test were used for *a posteriori* comparisons of measurements taken at different times of day.

Mean relative amount of scent emitted at different times was compared using ANOVA (repeated measures). Tukey tests were used for *a posteriori* comparisons between times of day. Non-parametric (Kruskal-Wallis) or parametric (ANOVA) tests were used after checking for normality (Kolmogorov-Smirnov test) and homoscedasticity (Levene's test) of data. Analyses were performed in the Software SPSS version 20 (IBM, Chicago, IL, USA).

RESULTS

Flower morphometry

Flowers of *G. perennis* are ca. 4-cm long. Their fused petals form a flower tube of 3.54 ± 0.36-cm long (mean ± SD), with an entrance height and width of 2.24 ± 0.32 cm and 2.27 ± 0.27 cm, respectively (Table 1). The analysed floral traits of *G. perennis* had a CV from 4% to 32% (Table 1). Stamen (first day) and pistil (first day) lengths were the most and least variable traits, respectively (Table 1). Measured floral traits were all positively correlated, except for length and opening height of the corolla. Nevertheless, only the traits width at corolla base and corolla length were significantly positively correlated (Pearson correlation, $r = 0.545$, $P < 0.01$). The same result was observed after principal components analysis: corolla length and corolla base width had high loadings for the first factor (Table 2), indicating they tend to covary. Corolla opening width was explained by the second component and corolla opening height by the third component (Table 2).

Flower anthesis

Flower anthesis was similar in plants from the two study sites. Mature buds started to unfold slowly during the night prior to flower opening, at around 20:00 h. At 05:00 h on the following day, 84% of the flowers were open. However, only at 07:00 h were all flowers fully open. Dehiscence of anthers occurred

Table 1. Descriptive statistics of floral morphological traits of *Gloxinia perennis*.

Floral trait	Mean ± SD (cm)	N	CV (%)
Corolla length	3.54 ± 0.36	32	9.77
Corolla base height	1.36 ± 0.09	32	7.46
Corolla base width	1.52 ± 0.17	32	11.19
Corolla opening height	2.24 ± 0.32	32	16.43
Corolla opening width	2.27 ± 0.27	32	9.96
Stamen length (1 day)	1.36 ± 0.10	16	7.37
Stamen length (2 day)	1.09 ± 0.35	16	32.18
Style length (1 day)	1.56 ± 0.07	16	4.18
Style length (2 day)	1.61 ± 0.20	16	12.29

Table 2. Factor loadings of the five floral morphological traits measured in *G. perennis* on the three-first principal factors derived from principal components analysis.

	Component		
Floral trait	1	2	3
Corolla base width	0.827	−0.001	−0.199
Corolla length	0.796	−0.042	−0.396
Corolla opening width	0.371	0.861	−0.013
Corolla base height	0.557	−0.665	0.332
Corolla opening height	0.344	0.247	0.872
% variance explained	37.69	24.95	21.36

during the day before anthesis, at around 22:00 h. The style stretched during the first hours of anthesis and the bilobate stigma reached its final position (*i.e.* horizontal and in contact with the anthers; Fig. 2) between 10:00 and 12:00 h on the first day. Although flowers seem to be protandrous, tests for stigma receptivity revealed that stigmas were already receptive 1 day before anthesis (bud phase). Therefore, partial herkogamy occurs, but only during the first hours of anthesis, when the bilobate stigma is still behind the anther openings. Flowers lasted for 1–2 days (mean ± SD: 1.62 ± 0.33 days, n = 25 flowers) when floral visitors were excluded. When exposed to floral visitors, most corollas abscised and fell off the plants on the same day on which they had opened.

Pollination success and breeding system

Natural pollination success in *G. perennis* was extremely high in Panguana, where 91.16% of the flowers (n = 262) set fruit. Although we lost data in Pozuzo due to the unexpected human interference, we observed that most of the flowers had developed fruits during the fieldwork period. We found significant differences in seed set among all the different treatments (Table 3; Kruskal-Wallis, $P < 0.01$). Bagged flowers for the spontaneous treatment set, on average, as much seed as the treatment of xenogamy and the control (Table 3; Mann-Whitney *U*-test with Benjamini-Hochberg correction, $P > 0.05$), but less seeds than the treatments of autogamy and geitonogamy (Table 3; Mann-Whitney *U*-test with Benjamini-Hochberg correction, $P < 0.05$). There were no significant differences in average seed set among all the hand-pollination treatments (*i.e.* autogamy, geitonogamy and xenogamy; Table 3; Mann-Whitney *U*-test with Benjamini-Hochberg correction, $P > 0.05$).

Flower visitors and effective pollinators

We recorded six bee species in flowers of *G. perennis*, four (*Apis mellifera*, *Eulaema cingulata*, *El. meriana* and *Scaptotrigona* sp.) in Pozuzo and four (*Euglossa imperialis*, *El. cingulata*, *El. meriana* and *Trigona* sp.) in Panguana (Table 4). In Pozuzo, we also recorded one beetle species (Chrysomelidae) on flowers of *G. perennis* (Table 4).

Three species of male euglossine bees (*El. cingulata*, *El. meriana* and *Eg. imperialis*) visited flowers of *G. perennis* to gather perfume. The floral visitation behaviour of these bees was consistent: bees approached a flower and hovered in front of it for a few seconds before landing. They then crawled up the flowers and at the base of the corolla tube they scratched the

Table 3. Seed set after the different treatments for the breeding system experiment with *Gloxinia perennis* flowers. Distinct letters indicate significant difference between treatments (Mann-Whitney *U*-test with Benjamini-Hochberg correction, $P < 0.05$).

Breeding system treatment	Sample size (n)	Seed set (mean ± SD)	Statistics
Spontaneous	8	11,062 ± 2,960	A
Control	9	12,012 ± 2,998	A
Autogamy	7	17,355 ± 3,475	B
Geitonogamy	10	16,191 ± 2,624	B
Xenogamy	6	13,945 ± 4,735	AB

Table 4. Floral visitors to *Gloxinia perennis* in Pozuzo and Panguana and type of resource collected.

Floral visitor (order/family/species)	Frequency observed[a]		Visitor behaviour[b]	Sex	Pollinator[c]
	Pozuzo	Panguana			
Hymenoptera					
Apidae					
Euglossini					
Euglossa imperialis	NO	I	Pe	♂	NP
Eulaema cingulata	R	V	Pe	♂	P
Eulaema meriana	I	V	Pe	♂	P
Apini					
Apis mellifera	F	NO	Po	♀	NP
Meliponini					
Scaptotrigona sp.	F	NO	Po	♀	NP
Trigona sp.	NO	F	Po	♀	NP
Coleoptera					
Chrysomelidae sp.	I	NO	Ft	♀♂	NP

[a]V = very frequent (>5 visits 15 min^{-1}); F = frequent (>1 but <5 visits 30 min^{-1}); I = infrequent (up to 1 visit 15 min^{-1}); R = rare (observed but no in the studied population); NO = not observed.
[b]Pe = perfume gathering; Po = pollen collection; Ft = flower tissue feeding.
[c]NP = non-pollinator; P = pollinator.

osmophore tissue with the dense tufts of hairs on the basal tarsi of their front legs. While entering the flowers, large-sized bees of *El. cingulata* and *El. meriana* (max. body height 0.85 and 0.95 cm, respectively; Fig. 3) contacted the stigma and then the anthers with their head and thorax, but medium-sized bees of *Eg. imperialis* (max. body height 0.52 cm) did not. During this movement, *Eulaema* bees deposited pollen grains on the stigmatic surface and more pollen became attached to their dorsum (mainly head and thorax; Fig. 3). After some seconds (mean ± SD: 12.68 ± 6.65 s, n = 28 visits; and 12.68 ± 5.26 s, n = 75 visits, in *El. cingulata* and *El. meriana*, respectively; Fig. 3) of perfume gathering, bees backed out of the flowers and transferred the perfume from the front- to the mid- and finally hind-legs while hovering in front of the flower. *Eulaema* bees normally visited the same flower two to four times before leaving. Although we did not mark bees individually, we frequently observed them moving among flowers of different individuals of *G. perennis*.

Besides male euglossine bees, workers of eusocial *Apis mellifera*, *Scaptotrigona* sp. and *Trigona* sp. also visited flowers of *G. perennis*. These bees collected pollen from anthers of

Fig. 3. Pollinators of *Gloxinia perennis*. Male *Eulaema meriana* approaching (A), landing on (B) and crawling into (C) a flower of *G. perennis*. *Eulaema cingulata* male hovering in front of a flower (D). Note the pollen load on the thorax and head of the bee (A, B, D) and the contact between *El. meriana* and the reproductive parts of *G. perennis*. Photographs by Paulo Milet-Pinheiro (A) and Günter Gerlach (B, C, D). Bar = 2 cm.

G. perennis (Table 4); they directly landed on anthers and left the flowers after a few seconds of pollen collection. While handling anthers, workers of these three species contacted the stigma. The beetle species (ca. 4-mm long) was observed feeding on pollen and corolla tissue of *G. perennis*, but never contacting the stigma of flowers. Beetles remained on flowers for long periods, frequently longer than a couple of hours.

Activity of euglossine bees at both Pozuzo and Panguana started soon after sunrise, at 06:00 h, and lasted until approximately 13:00 h (Fig. 4); whereas other flower visitors (*i.e.* stingless bees, honeybees, beetles) where sporadically observed until 16:00 h (data not shown). The recorded frequency of euglossine bees was very different between Pozuzo and Panguana (Fig. 4). Although *El. cingulata* males were also observed in the population from Pozuzo, none was recorded during our observation on focal flowers. Furthermore, the rate of *El. meriana* males visiting *G. perennis* flowers from Pozuzo was extremely low (Fig. 4). *Eulaema meriana* males from Pozuzo were only recorded from 07:00 to 10:00 h, with a peak of visits between 09:15 and 09:30 h (mean ± SD: 0.5 ± 0.93 visits; Fig. 4), whereas their peak in Panguana was between 11:15 and 11:30 h (16.64 ± 12.33 visits; Fig. 4). Visits by *El. cingulata* males in Panguana were most frequent between 08:15 and 8:30 h (16.92 ± 6.27 visits; Fig. 4), but none were recorded in Pozuzo during the time intervals measured and only one was observed in the surrounding area. *Euglossa imperialis* males were observed only in Panguana, with a peak of activity between 09:15 and 09:30 h (0.42 ± 1.44 visits; Fig. 4).

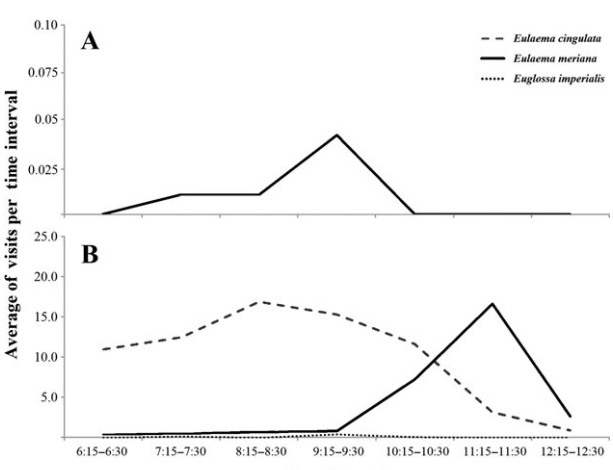

Fig. 4. Mean of visits per time interval (hours of day) per group of three flowers of *Gloxinia perennis* in Pozuzo (A, n = 8 flower groups) and Panguana (B, n = 24 flower groups). Time intervals from 12:30 h onwards are not shown since no further visits were observed after this time.

Chemical characterisation of floral perfume of *G. perennis*

In the chemical analyses of headspace samples of *G. perennis* flowers, we detected 16 compounds, of which 15 were identified (Table 5; see Table S1 for chemical characterisation of all headspace samples). Monoterpenes were by far the most representative substance class, with 13 different compounds. Besides monoterpenes, we recorded one aromatic (1,4-dimethoxybenzene) and one N-bearing compound (indole). (*E*)-Carvone epoxide (41%), limonene (23%), carvone (11%) and (*E*)-limonene epoxide (9%) were the major compounds, which together made up about 85% of the total floral perfume emitted by *G. perennis* flowers.

Daily fluctuation in floral perfume emission

The mean relative amount of perfume emitted at different times of day varied significantly (ANOVA repeated measures:

Table 5. Mean relative amount of volatile compound (n = 10) in the floral perfume bouquet of *Gloxinia perennis*. Volatiles are listed according to class and retention index (RI), calculated from retention times in relation to those of a series of n-alkanes separated on a non-polar DB-5 capillary column. Only the samples from 09:00 h are included here.

List of compounds	RI	Mean (%)	SD
Aromatics			
1,4-Dimethoxybenzene[a]	1,164	0.02	0.06
Monoterpenes			
Sabinene[a]	977	2.28	1.58
β-Pinene[a]	980	0.31	0.51
Myrcene[a]	993	2.70	0.88
Limonene[a]	1,032	23.37	7.64
1,8-Cineole[a]	1,034	4.00	1.14
Terpinolene[a]	1,088	0.16	0.22
6,7- Epoximyrcene[b]	1,093	1.74	0.67
(*Z*)-Limonene oxide[b]	1,134	1.63	0.23
(*E*)-Limonene oxide[b]	1,139	9.30	1.45
(*Z*)-Dihydrocarvone[b]	1,204	0.72	0.14
Shisofuran[b]	1,202	0.24	0.22
(*E*)-Dihydrocarvone[b]	1,205	0.97	0.23
Carvone[a]	1,250	10.75	2.75
(*E*)-Carvone epoxide[a]	1,277	41.05	8.48
N-containing compound			
Indole[a]	1,298	0.13	0.19
Unknown	1,332	0.63	0.71

[a]Identification based on authentic standards.
[b]Identification based on MS match with library entries and published RI.

$F_{3,27} = 55.68$, $P < 0.001$). *A posteriori* comparisons revealed that perfume emission increased significantly from 06:00 to 09:00 h, remained constant at 12:00 h, then decreased significantly at 15:00 h (Fig. 5).

DISCUSSION

The morphometric analyses in *G. perennis* flowers reveal that the corolla base width and corolla length covary; the observed correlation might be the result of pollinator selection as both directly influence behaviour of *Eulaema* males while gathering perfume from the osmophore. Overall, the flower morphology of *G. perennis* seems to be an adaptation to pollination by euglossine bees, which is unusual within the hyper-diverse *Gesneriaceae*. Indeed, among *Gloxinia* species (*i.e. G. alterniflora* A.O. Araujo, *G. erinoides* (DC.) Roalson & Boggan, *G. perennis* and *G. xanthophylla* (Poepp.) Roalson & Boggan; Araujo *et al.* 2010), only *G. perennis* and *G. alterniflora* present campanulate flowers, an osmophore and lack of any nectary; whereas *G. erinoides* and *G. xanthophylla* have tubular or infundiliform flowers, with an annular nectary and no osmophore (*G. erinoides* might also be fragrant). The latter two of the 34 species seem to be pollinated by nectar-collecting bees, whereas *G. alterniflora* might be also pollinated by perfume-collecting euglossine bees. Unfortunately, no data on pollination of any other *Gloxinia* species are currently available. In any case, the flower morphology of *G. perennis* seems to be a result of adaptive evolution, since the species is derived within the *Gloxinia* genus (Roalson *et al.* 2005).

Herkogamy and protandry are common among *Gesneriaceae* species (Wiehler 1983; Gao *et al.* 2006). Flowers of *G. perennis* were also found to be protandrous (Vogel 1966; Witschnig *et al.* 2008); however, in the populations that we investigated, this was not corroborated. *Gloxinia perennis* is indeed partially herkogamous during the first hours of anthesis, but protandry does not occur as the stigma is already receptive when the anthers dehisce. Flowers of *G. perennis* last 2 days in the absence of pollinators, but less than 1 day when pollinators visit flowers. When pollinators are scarce for *G. perennis*, spontaneous delayed selfing develops as a 'just in case" strategy (Eckert & Schaefer 1998; Navarro *et al.* 2007); this is evident in Pozuzo where pollinator visits are extremely low but fruit production was nevertheless high (C. Martel, personal observation). The stigma, in its final position, is ahead and slightly in contact with the dehiscent anthers (see position of stigma and anthers in Fig. 2); this results in deposition of self-pollen on the stigma. This mechanism of spontaneous self-pollination, in which the stigma, style and stamens move, is the most common among angiosperms (Freitas & Sazima 2009). The self-pollination process is boosted when the corolla starts to detach from the calyx and the filaments twist their position.

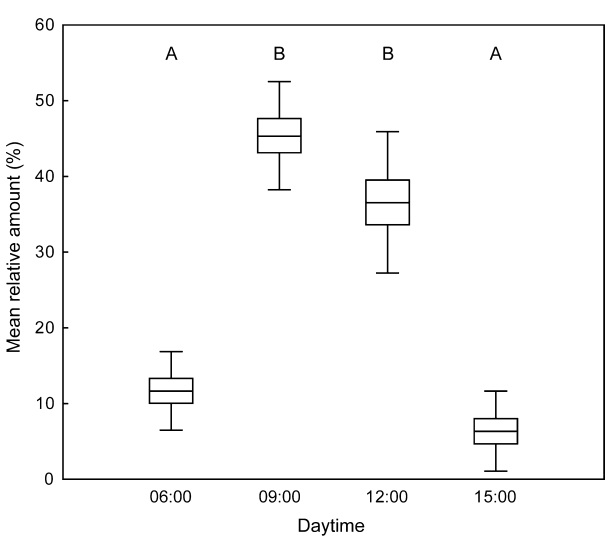

Fig. 5. Mean relative amount of perfume emitted by flowers of *Gloxinia perennis* at different times of day. Different letters indicate significant differences ($P < 0.05$) in the perfume emission between times of day (ANOVA repeated measures followed by Tukey test).

The breeding system experiments showed that *G. perennis* is self-compatible and also outcrossing. Although spontaneous self-pollination occurs, we cannot rule out that apomixis also occurs. Spontaneous self-pollination is known in other *Gesneriaceae* but is rare within the family (see Fenster & Martén-Rodríguez 2007; Camargo *et al.* 2011). Seed set was significantly higher in the autogamy treatment than in spontaneous-pollinated flowers or flowers naturally visited by pollinators, which indicates that seed set in natural populations is pollen-limited. Cross- and self-pollination occur in *G. perennis* flowers, suggesting that this species has evolved a mixed mating strategy. This might be explained by its wide distribution area, as well as the short lifespan of its flowers, both of which are aspects that might reduce the chance of pollinator visits, which favour the evolution of spontaneous selfing (Lloyd 1992; Freitas & Sazima 2009). This is in contrast to the *Eulaema*-pollinated *Catasetum* species, which present flowers that last several days (if not pollinated) and in which self-pollination is impossible because the flowers are unisex. In Pozuzo, plants might also benefit from self-pollination, since euglossine diversity and abundance is lower at higher altitudes (Roubik & Hanson 2004) and, therefore, pollination services might be scarce. Nevertheless, the spontaneous self-pollination strategy might only play a role in the absence of pollinators. Thus, cross-pollination is prioritised by *G. perennis* as partial herkogamy occurs and flowers last only 1 day when pollinators are present, reducing chances of the stigma contacting directly with pollen from the anthers.

In our study, we found several insects visiting flowers of *G. perennis*. However, only *Eulaema* species (*i.e. El. cingulata* and *El. meriana*) have the appropriate body size and behaviour to contact the anthers and stigma, thereby promoting pollination. The *Euglossa* bees are too small to contact the stigma or anthers when visiting the flower to gather perfume and, therefore, do not pollinate flowers. Although honeybees and stingless bees may contact the stigma and anthers during their visits, their small size and behaviour (*i.e.* pollen is loaded on hind legs) prevent transfer of pollen to the stigma. Honeybees and stingless bees also enter the flowers upside down during the first hours of anthesis, when the stigma has not yet reached the anthers, and they contact the anthers with the legs only; all of which make the transfer of pollen very unlikely. Furthermore, stingless bees usually make holes in buds in order to remove pollen; they even leave the patch after visiting a single flower. Conversely, *Eulaema* bees receive large pollen loads on the thorax and head during their perfume gathering (see Fig. 3); after

visiting a flower, they frequently fly to other flowers of the same patch to collect more perfume and promote pollination by depositing pollen on the stigmatic surface. Outside of the natural distribution of *G. perennis*, *El. meriana* has been recorded on flowers in Costa Rica (Vogel *et al.* 2005; Witschnig *et al.* 2008) and Panama (Dressler 1968), whereas *El. nigrita* was reported visiting this species in Brazil (Vogel 1966). This emphasises the importance of *Eulaema* species as pollinators of *G. perennis*. *Eulaema meriana* is a widely distributed species (Oliveira 2007) that matches the natural distribution of *G. perennis* in the Amazon Basin, as does *El. cingulata* (Oliveira 2007). *Eulaema* bees, and euglossine bees in general, have a large flight capability (Kroodsma 1975; Wikelski *et al.* 2010) and are known as excellent long-distance pollinators (Janzen 1971; Dressler 1982), which would favour cross-pollination over distance in the patchily distributed *G. perennis*.

The strongly scented flowers of *G. perennis* have long aroused the curiosity of naturalists, *e.g.* Carl Linnaeus (1753) and Herman Crüger (1864); however, their chemical composition remained unknown until the report of Gerlach & Schill (1991). These authors pointed out that *G. perennis* flower perfume contains carvone and carvone epoxide. Indeed, Gerlach & Schill (1991) listed at least 13 compounds present in the floral perfume bouquet of a single cultivated *G. perennis* individual, in which (*E*)-carvone epoxide accounted for 23% of the total perfume emitted. Together, the four major constituents (*i.e.* (*E*)-carvone epoxide, 1,8-cineole, limonene and methyl salicylate) accounted for more than 70% of the total perfume emitted. Similarly, we found (*E*)-carvone epoxide and limonene as major constituents of *G. perennis* and, together with another two compounds (*i.e.* carvone and (*E*)-limonene oxide), accounted for more than 80% of the total perfume bouquet. Nevertheless, 1,8-cineole was a minor constituent in our samples and methyl salicylate was not detected. All the major constituents of the floral perfume of *G. perennis* have also been recorded in other *Eulaema*-pollinated species belonging to distant angiosperm genera (*e.g. Anonnaceae*, *Orchidaceae*, *Euphorbiaceae*; Gerlach & Schill 1991; Milet-Pinheiro & Gerlach 2017; Teichert *et al.* 2009; Whitten *et al.* 1986). Among such compounds, carvone epoxide seems to be especially important in the attraction of *Eulaema* species, as field baiting experiments with synthetic (*E*)-carvone epoxide attracted *Eulaema* males (Whitten *et al.* 1986), and the presence of this compound seems to be universal in *Eulaema*-pollinated *Catasetum* species (Milet-Pinheiro & Gerlach 2017).

Males of both *Eulaema* species visit the flowers of *G. perennis* during the first hours of daylight. However, activity of *El. cingulata* males peaks between 07:00 h and 10:00 h, but they later compete for the reward with the bigger males of *El. meriana*. A change of visitors during daytime was already reported by Witschnig *et al.* (2008), in which *El. meriana* males only collect perfume during the first hours of the day (*i.e.* 05:00–07:00 h) and are later replaced by *Euglossa* species. *Eulaema meriana* males have different times of activity peaks on *G. perennis* from Panguana and Pozuzo. In Panguana their peak activity was later than in former reports for perfume-rewarding flowers (Dodson 1978; Janzen 1981; Carvalho & Machado 2002; Witschnig *et al.* 2008), although this might be more related to their task preferences (*i.e.* harvesting perfume from other sources, seeking for nectar, etc.) as *El. meriana* males were observed to be active at dawn in Panguana (C. Martel, personal observation). Furthermore, it is known that the peak of activity of some *Eulaema* species on perfume-rewarding flowers is not restricted to the early morning, and that perfume-gathering activity can be extended until 09:00 h (*e.g.* Carvalho & Webber 2000) or even reach a peak at midday. For example, *El. bombiformis* has peak activity on *Unonopsis stipitata* Diels (*Anonnaceae*) between 12:00 and 13:00 h (Teichert *et al.* 2009). Nevertheless, the rule seems to be that *Eulaema* bees take advantage of their size to displace the smaller competitors (*e.g. Euglossa* bees; Teichert *et al.* 2009), even between large and smaller *Eulaema*. Thus, for example, the bigger *El. meriana* males progresively displace *El. cingulata*, as found for *El. bombiformis* males do on *Eg. imperialis* (Teichert *et al.* 2009). Witschnig *et al.* (2008) proposed that *Eulaema* males arrive earlier to flowers of *G. perennis* than *Euglossa* males to have an advantage in obtaining the perfume reward. However, this does not seem to be true among *Eulaema* males, as big *Eulaema* easily overcome smaller flower competitors and perfume in *G. perennis* is produced constantly by the flowers throughout the morning.

Flowers of *G. perennis* from both Pozuzo and Panguana were perceived to emit more perfume in the morning than in the afternoon, as evaluated with human olfaction. In Panguana, flowers were perceived to emit a strong perfume from 06:00 to 12:00 h, unlike the population at Pozuzo, where perfume emission was only perceived from 09:00 to 12:00 h (C. Martel, personal observation). This was corroborated by chemical analysis of floral perfume samples from plants in Pozuzo, where the peak of perfume emission occurs between 09:00 and 12:00 h, and our preliminary analysis of samples from Panguana, where the peak emission occurs between 06:00 and 12:00 h (data not shown). Overall, the time of highest perfume emission of *G. perennis* in Pozuzo matches well with the time of higher activity of *Eulaema* bees on its flowers, and this seems to be true also for the population in Panguana. A similar pattern was already recorded in *C. uncatum*, which is pollinated by *Euglossa* males (Milet-Pinheiro *et al.* 2015). Daily fluctuations in perfume emission are assumed to be a strategy to save energy when activity of pollinators is low (Armbruster & McCormick 1990; Milet-Pinheiro *et al.* 2015; Milet-Pinheiro & Gerlach 2017). In addition, by producing less perfume when pollinators are less active in perfume harvesting, plants may also benefit by being less conspicuous to natural enemies, as suggested for other perfume-rewarding plants such as *Dichaea pendula* (Aubl.) Cogn. (Nunes *et al.* 2016), without losing relevant pollinator services.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Absolute (peak area) and relative amount (peak area of individual compounds in relation to the sum of all compounds in a given sample) of floral scent compounds.

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