ORIGINAL ARTICLE

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The reproductive biology of two understory plants in the Atlantic rain forest, Brazil

Abstract Outcrossing and sexual reproduction of most flowering plants depends on pollinators. Plant traits likely to be involved in pollinator attraction include flower color, shape, and size. Furthermore, plant or flower density and the temporal flowering pattern may have an effect on reproduction. In this study, we examine the pollination ecology, breeding system, female reproductive output, and germination of two tropical understory species, *Stenostephanus lobeliiformis* (Acanthaceae) and *Besleria melancholica* (Gesneriaceae), which differ in these traits. Pollinator observations revealed that the dense flowering *S. lobeliiformis* with pinkish flowers received a higher diversity of pollinators, but visitor frequency measured as visits per flower per hour was much less ($0.1\ h^{-1}$) than that to *B. melancholica*, which has a smaller floral display of dull-colored flowers ($1.5\ h^{-1}$). Pollination experiments revealed that *S. lobeliiformis* but not *B. melancholica* is pollen-limited. In addition, both species are partially self-incompatible and depend on pollinators for outcrossing. Natural fruit set of open-pollinated unmanipulated flowers (control treatment) in both species is 22–26 %. Germination studies indicated inbreeding depression in *S. lobeliiformis*. We conclude that the pollination ecology of these species is influenced by a broad set of traits and that very different combinations of these traits can be successful in terms of reproduction.

Keywords *Besleria melancholica* · Floral visitors · Hummingbird · Nectar · Nectar robbing · *Phaethornis* · Pollination experiments · Reproductive biology · *Stenostephanus lobeliiformis* · *Trigona*

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Introduction

Most flowering plants depend on animals for effective pollination and sexual reproduction (Buchmann and Nabhan 1996). Plant traits likely to be involved in pollinator attraction include, for example, flower color, shape, and size (Faegri and van der Pijl 1979; Sutherland and Vickery 1993). The principle reward for most flower visitors is nectar (Proctor et al. 1996). It has been frequently proposed that nectar sugar compositions represent adaptations to dietary preferences of the respective pollinators and thus form part of the pollination syndrome (Baker et al. 1998; Schmidt-Lebuhn et al. 2007). Furthermore, floral density may have an effect on reproduction (Kunin 1993; Ågren 1996). Understory plants in tropical rain forests are often patchily distributed, with densities ranging from dense aggregations of reproductive conspecifics to spatially isolated individuals (Kay and Schemske 2003; Bruna et al. 2004; Schleuning et al. 2008). Plants growing in dense floral neighborhoods of conspecifics or producing large numbers of new flowers each day over a short period (mass-flowering; Heinrich and Raven 1972) are often more attractive to pollinators. Augmented attractiveness likely attracts numerous and diverse flower visitors (Gentry 1974; Frankie et al. 1976), leading to an increased pollen deposition and fruit set (Kunin 1997; Roll et al. 1997; Burd 1994). However, at high densities, plants/flowers may also compete for pollination services, leading to reduced visitation rates per flower and a decreased fruit set (Weiner 1982; Rathcke 1983; Ghazoul 2005). Isolated plants or plants that produce only small numbers of new flowers over an extended period (steady state-flowering; Gentry 1974) usually attract few or low-quality pollinators (Jennersten and Nilsson 1993; Kunin 1997), possibly resulting in pollen limitation and low fruit set (Larson and Barrett 2000; Ashman et al. 2004).

Pollen limitation may not only result from insufficient pollen transfer (quantity limitation) but also from deposition of low-quality pollen (quality limitation),

such as genetically related, incompatible pollen (Aizen and Harder 2007). Pollen quality effects associated with both self-fertilization and mating between related plants can additionally reduce seed production, which is a sign of inbreeding depression (Aizen and Harder 2007).

Herbaceous plants of the understory of neotropical forests constitute an important component of plant diversity (Gentry and Dodson 1987), representing 20–50 % of the local diversity of vascular plants (Andreata et al. 1997). However, there is a lack of knowledge on the ecology, in particular pollination and reproductive biology, of these understory species (Freiberg and Gottsberger 2001; Drucker et al. 2008). The Atlantic rain forest (''Mata Atlântica'') of southeastern Brazil is a biodiversity hotspot with an outstanding level of endemism (Myers et al. 2000), which currently is highly threatened by habitat loss, deforestation, and fragmentation (e.g., Tabarelli et al. 2005; Ribeiro et al. 2009). Thus, basic knowledge on the reproductive ecology of important biodiversity components of this forest, such as understory species, might contribute to the understanding of ecosystem functions and services and might lead to implementation of conservation approaches.

This study deals with two understory species from the Atlantic Forest of southeastern Brazil. The species differ in many important reproductive traits: plant density, flowering phenology, floral morphology, and flower color. *Stenostephanus lobeliiformis* Nees (Acanthaceae) has scarlet narrow tubular flowers and grows in dense populations, with all individuals being highly synchronously mass-flowering. *Besleria melancholica* (Vell.) C.V. Morton (Gesneriaceae), in contrast, has white open tubular flowers. The species is patchily distributed with individuals showing the steady-state flowering pattern. While the female reproductive output in the mass-flowering species may be pollen-limited due to a high competition for pollination, the steady-state flowering species may also be pollen-limited due to low pollinator attraction. The objectives of the study were to investigate (1) the diversity and abundance of flower visitors (potential pollinators), (2) floral nectar sugar composition as an indicator of pollinator composition, (3) the influence of the floral display size on visitor number and frequency, (4) the breeding system and female reproductive success to indicate whether the species are self-compatible (or not) and pollen-limited, (or not), and (5) seed germination of both species as an initial look at inbreeding depression.

Materials and methods

Study area and species descriptions

The study was conducted from June to October 2009 and from May to August 2010 in the Atlantic rain forest (Mata Atlântica) of the state of Rio de Janeiro, Brazil, in the private reserve ''Reserva Ecológica de Guapiaçu'' (REGUA—22°25′53″S, 42°45′20″W). The 5,500-ha reserve is located on the south-facing slopes of the Serra dos Orgãos Mountain range, about 100 km from the city of Rio de Janeiro. The mean annual temperature for this region is about 23 °C with a mean annual rainfall of about 2,560 mm. The vegetation can be classified as an evergreen dense ombrophilous forest (Veloso et al. 1991) that is typical for the lower and medium elevations of the coastal mountain range (Morellato and Haddad 2000; Oliveira-Filho and Fontes 2000).

Besleria melancholica (Vell.) C.V. Morton (Gesneriaceae)

This species is a perennial sub-shrub of a height up to 1.50 m (mean height 1.20 m), endemic to the Atlantic rain forest of the state of Rio de Janeiro. The genus is common in the forest understory in shady habitats near slopes and paths (Lopes et al. 2005; Kriebel-Haehner 2006). The hermaphroditic, protandrous, scentless flowers are tubular; the 20–25-mm-long corolla is of white color with a rather wide opening of 6–9 mm. The calyx is bright yellow. The axillary inflorescences consist of approximately eight flowers. Usually, only one to six (mean four) open flowers are presented per individual at the same time. Each flower remains open and viable for 8–13 days, a single individual plant bears flowers over a period of about 4 mo from late June until October (steady-state flowering). Four to six weeks after anthesis, fleshy, berry-like, orange-colored fruits with thousands of tiny seeds are formed, which are dispersed by birds. *B. melancholica* has a patchy distribution, with a mean number of 3.67 ± 1.61 flowering individuals and 18.6 ± 11.1 open flowers on average per plot of 2×5 m size (*n* plots = 3).

Stenostephanus lobeliiformis Nees (Acanthaceae)

This species is an erect perennial ramified herb that can reach a height of up to 1.50 m. The terminal inflorescences of 10–22 cm in length consist of 18–40 flowers. The ornithophilous, hermaphroditic, protandrous, scentless flowers are vivid scarlet in color and the corolla has an 18-mm-long tube with a narrow opening of 2 mm. The two stamens and the stigma exceed the corolla by 7–11 mm without any spatial separation (no herkogamy). Floral anthesis (per flower) lasts for 3–4 days; the whole inflorescence flowers for approximately 4 weeks. All individuals in the habitat flower annually over a period of 4–5 weeks between late June and August (mass flowering). The fruits are dry capsules; the seeds (maximum four per fruit) are dispersed autochorously. This species grows in dense populations of 0.4–0.6 ha in size near river sites (Lindner et al. 2010) with a mean ($\pm$SD) number of 11 ± 3.65 flowering individuals and 80.4 ± 30.4 open flowers on average per 2×5-m plot (*n* plots = 3, repeated measure in 2009 and

2010 in the same plots). The distribution of the genus is exclusively neotropical and ranges from southern North America to Colombia and Brazil. *S. lobeliiformis* is listed as a vulnerable species in the state of Espirito Santo in Brazil (Daniel 1999).

The species are sympatric in some parts of their distributions, in particular in the Atlantic rain forest of the state of Rio de Janeiro. The species were sympatric in two study sites, where *S. lobeliiformis* was dominant; only one or two individuals of *B. melancholica* were growing and flowering nearby.

Statistical analysis

All analyses are described in each of the following sections in the methods. They were carried out using SigmaStat 3.0.1, SPSS Inc. 2003.

Observation of flower visitors

Flower visitors were observed for 10 (*S. lobeliiformis*) and for 14 days (*B. melancholica*), respectively, continuously from 8h00 to 16h00 in 2009 and 2010 (June until August), resulting in 80 (*S. lobeliiformis*) and 112 (*B. melancholica*) h of observation in total. Care was taken that the weather conditions at all observation days were similar (sunny with up to 50 % clouds, no rain) and thus comparable. For each species, flower visitor observations were carried out at three different plots of varying density of flowering conspecifics. The observation plot size was 2×5 m. Each plot was located within a different population of conspecific plants. The average distance between plots/populations was approximately 2.75 km. The same plots were observed several times. Each observation day, all flowering conspecifics and number of open flowers per plot were counted to test the impact of the floral display size on the number/frequency of flower visitors. Flower visitors entering an open flower or touching the anthers/stigma were counted as legitimate visitors. When piercing the corolla to steal nectar without touching reproductive parts of a flower (primary nectar robbers) or using already existing holes made by primary nectar robbers (secondary nectar robbers) they were recorded as nectar robbers (definition following Inouye 1980) and excluded from further analysis. We observed stingless bees (Meliponini) of the genus *Trigona* being primary and secondary nectar robbers as well as the hermit hummingbird *Phaethornis ruber* at times being a secondary nectar robber. Each time a flower visitor entered the observation plot it was counted. When a visitor left the plot and entered it again, it was counted as a new visitor. Species of flower visitors were determined when possible. Hummingbird species were identified using a field guide (Endrigo 2006) and confirmed by ornithologists from the University of Rio de Janeiro. The genus of the stingless bee species

were identified with an identification key for neotropical insects (Hogue 1993).

In order to test whether the different observation plots are comparable in terms of the recorded total number of visits and the number of visits for each visitor group, we performed two separate one-way ANOVAS in both plant species (ANOVA 1: factor "plot" vs. response variable "total number of visits": ANOVA 2: factor "plot" vs. "number of visits of each visitor group"). Due to non-normally distributed data of *S. lobeliiformis*, we calculated Kruskal–Wallis one-way ANOVAs on ranks. In case of *B. melancholica*, data were normally distributed, thus one-way ANOVAs were performed. To analyze whether the abundance of the single visitor groups significantly differ from each other, e.g., if there are more hummingbirds or Hymenoptera visits in general, a Mann–Whitney rank-sum test was carried out due to non-normally distributed data of *B. melancholica* and a Kruskal–Wallis one-way ANOVA on ranks was used for the data of *S. lobeliiformis*. To test for an interrelation between the floral display size and total visitor frequency, a linear regression was performed in both species. Frequency data underwent log-transformation to normalize them.

Nectar sugar composition

Twenty nectar samples of *B. melancholica* and *S. lobeliiformis* each were collected in the late afternoon from flowers that were bagged before anthesis using micro-pipettes (Drummond "Microcaps" 10 μl). First the nectar amount was noted then the nectar was transferred to small strips of filter paper, which were then dried and stored in silica gel (Schwerdtfeger 1996). The laboratory analysis was carried out with a "Merck Hitachi-HPLC" with RI-Detector. The solvent used was 30 % water and 70 % acetonitrile (ACN); the column was a Macherey–Nagel Nucleodur 100-5 NH2, 250×4.6 mm. S/H ratio was calculated as [sucrose]/[fructose + glucose].

Investigation of the breeding system

To determine the breeding systems of the species and to test for self compatibility and potential pollen limitation, we performed controlled pollination treatments according to Dafni (1992). In the case of *B. melancholica*, pollination experiments were carried out at all available flowering individuals scattered in the forest (between 80 and 420 m a.s.l.), due to the need of a sufficient number of flowers (see sample sizes in Table 1). Pollination experiments of *S. lobeliiformis* were carried out at three sites in the forest, which are located between 240 and 420 m a.s.l. with a minimum of 1.5 km distance and a maximum of 4 km distance between each other. Within these sites, randomly chosen individuals were subjected to the different pollination treatments or designated as

Table 1 Sample size of the pollination experiments of *Besleria melancholica* and *Stenostephanus lobeliiformis* (*n* number, *inflors.* inflorescences, *AG* agamospermy, *SS* spontaneous selfing, *HS* hand selfing, *OC* outcrossing, *HC* hand crossing, *C* control), and number of fruits

Treatment	Plants (*n*)	Inflors. (*n*)	Flowers (*n*)	Fruits (*n*)
B. melancholica				
AG	4	33	76	0
SS	4	37	92	2
HS	5	41	79	7
OC	4	41	131	56
HC	7	75	160	49
C	5	67	167	41
S. lobeliiformis				
AG	14	26	541	5
SS	16	35	531	25
HS	14	34	526	52
OC	24	62	1,367	278
HC	24	55	1,118	350
C	26	60	1,177	290

Sample sizes of *B. melancholica* are low due to few flowering individuals in the study area

control plants. The number of plants per treatment was equal at all three sites. Nevertheless, some treated individuals died due to fallen trees or were damaged following storms and heavy rain.

The pollination treatments were conducted from June to September 2009 (*B. melancholica*) and from May to July 2010 (*S. lobeliiformis*). The different treatments were carried out on separate individuals, e.g., all flowers or inflorescences produced by an individual were subject to the same treatment. Due to the fragile inflorescences and the small flowers of both species, bagging of single flowers was not possible or too risky in terms of damaging them. Thus, we decided to bag whole inflorescences or even plants, when required by the treatment. In the agamospermy treatment (AG), flowers were emasculated and the inflorescences shielded from pollination by bagging with pollination bags (Seedburo Equipment Company, Chicago, IL, USA) to test whether flowers set seed without fertilization. Emasculation took place before the anthers released pollen. Other flowers were emasculated and either self-pollinated with pollen from other flowers of the same plant (hand selfing, HS, geitonogamy) or cross-pollinated with pollen from another individual (hand crossing, HC) and then bagged to exclude pollinators. Pollen for the hand-crossing treatments was collected within the same population in case of *S. lobeliiformis* with a minimum distance of 20 m to the treated individuals to decrease the likelihood of crossings between closely related individuals. In the case of *B. melancholica*, pollen was collected from all available flowers in male-phase from different patches in the whole study area of the nature reserve due to the low number of flowering individuals. In all pollen supplementation treatments, care was taken that each stigma was fully covered with conspecific pollen. To investigate cross-pollination under natural conditions (outcrossing, OC) flowers were emasculated to prevent selfing and left un-

bagged. Experiments to determine whether intact flowers yield fruit in the absence of pollinators (spontaneous selfing, SS) were performed by bagging all inflorescences with flowers still in bud stage and leaving them undisturbed until fruit formation. Open-pollinated flowers were unmanipulated to serve as controls (C) for natural pollination. Furthermore, we calculated the index of self-incompatibility (ISI) as the average number of seeds per fruit by self-pollination divided by the average number of seeds per fruit by cross-pollination (Zapata and Arroyo 1978; Schmid et al. 2011).

Female reproductive success

Four to six weeks (*B. melancholica*) and 2–3 weeks (*S. lobeliiformis*) after flowering, mature fruits of the treated individuals were collected. We recorded the total number of fruits per plant and treatment as well as the number of intact fruits and those damaged by herbivores or fungi. In the following studies, only intact fruits were used. Of these intact fruits, seeds were extracted and all seeds per fruit were weighed to the nearest 0.001 g. Because of the positive interrelation between fruit weight and seed mass per fruit (*B. melancholica*: $R^2 = 0.859$, $P < 0.001$, $n = 29$; *S. lobeliiformis*: $R^2 = 0.484$, $P < 0.001$, $n = 118$; linear regression), we used seed mass per fruit as a surrogate of reproductive success. Thus, only data of the seed mass per fruit are included in the results. The fruit set per plant was estimated by the number of mature fruits divided by the total number of flowers of an individual. A measure of whole-plant seed production was calculated by multiplying mean fruit set by mean seed mass per fruit. We calculated the pollen limitation index (PLI) as (1—(open (C)/pollen-supplemented (HC) whole plant seed mass)) after Larson and Barrett (2000) and Martén-Rodríguez and Fenster (2010). Two-way ANOVAs (factors: site, treatment; response variable: fruit set or seed mass per fruit, respectively) were carried out followed by a Student–Newman–Keuls pairwise comparison procedure to test for potential differences of the fruit set and seed mass per fruit according to each treatment separately. We separated two groups for comparison: outcrossing treatments (HC and OC) and selfing treatments (SS and HS). A Mann–Whitney rank-sum test was then performed to test whether outcrossing treatments differ significantly from selfing treatments in fruit set and seed mass per fruit.

Germination experiments

Seed viability was investigated through germination studies. The number of plants from which seeds were extracted per treatment is as follows: *B. melancholica*: 2 (SS), 4 (HS), 4 (OC), 7 (HC), 5 (C); *S. lobeliiformis*: 5 (SS), 4 (HS), 16 (OC), 20 (HC), 20 (C). In both species, seeds resulting from the same treatment were mixed in equal parts. Seeds that were damaged or had signs of predation

were excluded from germination studies. In case of *B. melancholica*, we put 50 seeds each on filter paper in Petri dishes (moistened with distilled water) and stored them in conditioning cabinets (32 °C day/20 °C night, 12 h light/12 h dark). We used 12 replicates for each treatment, which represents in total 600 seeds per treatment. The spontaneous-selfing treatment (SS) resulted in a low number of seeds, thus only six replicates were available for the germination study. The agamospermy treatment (AG) resulted in no seed production. Seed germination of *S. lobeliiformis* was tested the same way as described above. Depending on the number of seeds available (seed production per treatment), we had 11 to 18 replicates of 30 seeds each per Petri dish and treatment. Only 17 seeds each were available from the spontaneous-selfing (SS) and hand-selfing (HS) treatments. Germination was checked every second day and counted as positive when the radicle broke through the testa. The germination study was completed after 70 days, when no more seeds had germinated for several days.

To test whether seed germination differs among the different treatments, we performed a Kruskal–Wallis one-way ANOVA on ranks (non-normally distributed data; factor: treatment, response variable: germination) in both species.

Results

Flower visitors and nectar sugar composition

Both the number of total legitimate visits and the number of visits of each visitor group at all observation plots were homogenous in both plant species (total visits vs. plot, *S. lobeliiformis*: $H = 2.43$, $P = 0.36$; plot vs. n Lepidoptera $H = 1.5$, $P = 0.85$; plot vs. Hymenoptera $H = 0.93$, $P = 0.68$; plot vs. Trochilidae $H = 0.49$, $P = 0.88$; *B. melancholica*: total visits vs. plot $F = 3.50$, $P = 0.09$; plot vs. Lepidoptera $H = 2.61$, $P = 0.28$, plot vs. Hymenoptera $H = 3.01$, $P = 0.12$; plot vs. Trochilidae $H = 1.44$, $P = 0.25$). Thus, the plots were comparable.

We noted 17 different legitimate flower-visitor species to the flowers of *S. lobeliiformis* (Table 2). The most frequent legitimate visitor was *Trigona* sp., a stingless bee (Hymenoptera, Meliponini). All five observed Hymenoptera visitor species together accounted for 88.8 % of all legitimate visits to *S. lobeliiformis* (Table 2). Significantly more visits by Hymenoptera than by all other visitor species were recorded (Hymenoptera vs. Trochilidae, Lepidoptera, Coleoptera: each $q \geq 11$, $P < 0.001$). However, about 50 % of all visits of *Trigona* bees to *S. lobeliiformis* were as primary or secondary nectar robbers. Four different species of nectar-feeding hummingbirds accounted together for 5.5 % of all legitimate visits, and butterflies accounted for 4.3 % of all legitimate visits (Table 2). In the case of *B. melancholica,* only four different legitimate flower-visitor species were observed, and three hummingbird species accounted together for 87 % of all legitimate visits

Table 2 Visitor diversity, legitimate visits in %, and mean visitor number per flower h^{-1} (frequency) by all observed legitimate visitor species to the flowers of both plant species for each three observation plots of 2 × 5 m and the total observation period (*Besleria melancholica*: 112 h; *Stenostephanus lobeliiformis*: 80 h)

Legitimate flower-visitor species	Visits (%)	
	B. melancholica	*S. lobeliiformis*
Trochilidae in total	**87**	**5.5**
Thalurania glaucopis	1	46
Aphantochroa cirrochloris	2	32
Phaethornis ruber	97	20
Phaethornis squalidus	0	2
Hymenoptera in total	**13**	**88.8**
Meliponini (stingless bees) *Trigona* sp.	100	96
Vespidae (wasps) one unidentified species	0	2
Three unidentified bee species	0	2
Lepidoptera in total	**0**	**4.3**
Danainae one unidentified species		69
Three unidentified species		31
Diptera in total	**0**	**1.3**
Three unidentified species		
Coleoptera in total	**0**	**0.1**
One unidentified species		
Total number of visitor species	4	17
Mean visitor number per flower h^{-1}	1.5 (SD 1.17)	0.1 (SD 0.04)

Bold numbers express the legitimate visits in % by each visitor guild
SD standard deviation

(Table 2). Significantly more Trochilidae than Hymenoptera were observed ($T = 124$, $P < 0.001$). The stingless bee *Trigona* sp. was recorded as the only Hymenoptera visitor species at the flowers of *B. melancholica* and accounted for a total of 13 % of all legitimate visits. In this species, about 90 % of all visits of *Trigona* bees were as secondary nectar robbers.

The flowers of *S. lobeliiformis* were observed secreting a low amount of 5.52 ± 2.50 μl on average of nectar per day, whereas the flowers of *B. melancholica* secreted 33.66 ± 27.42 μl. The nectar of both species was found to be sucrose-dominant and diluted (total sugar concentration: 14.7–17 %), whereas it was poor in the hexoses glucose and fructose (Table 3).

Floral display, number of visits, and visitor frequency

With an increasing number of open flowers of *S. lobeliiformis* per plot, significantly more visits in total were observed ($R^2 = 0.68$, $P < 0.01$), as well as significantly more visits of Hymenoptera or Trochilidae (Hymenoptera: $R^2 = 0.55$, $P < 0.05$; Trochilidae: $R^2 = 0.46$, $P < 0.05$). No correlation between the floral display size and the number of visits could be found for *B. melancholica*, neither for the total number of visits ($R^2 = 0.018$, $P = 0.288$) nor for visits of hummingbirds ($R^2 = 0$, $P = 0.962$) or bees ($R^2 = 0.043$, $P = 0.214$). Mean visitor frequency to one flower of *S. lobeliiformis* was 0.1 h^{-1}

Table 3 Nectar sugar composition (mean ± standard deviation SD) of *Besleria melancholica* and *Stenostephanus lobeliiformis*

	Sucrose (%)	Fructose (%)	Glucose (%)	Sugar (g/ml)	Total nectar concentration (%)	Sugar ratio
B. melancholica ($n = 20$)	10.92 ± 5.90	3.69 ± 2.85	0.11 ± 0.74	0.15 ± 0.09	14.72 ± 8.68	3.35 ± 1.48
S. lobeliiformis ($n = 20$)	12.22 ± 3.62	2.71 ± 0.72	2.15 ± 0.57	0.17 ± 0.04	17.09 ± 4.38	2.56 ± 0.68

Sample size (n) is given for each species. Sugar ratio was calculated as [sucrose]/[fructose + glucose]
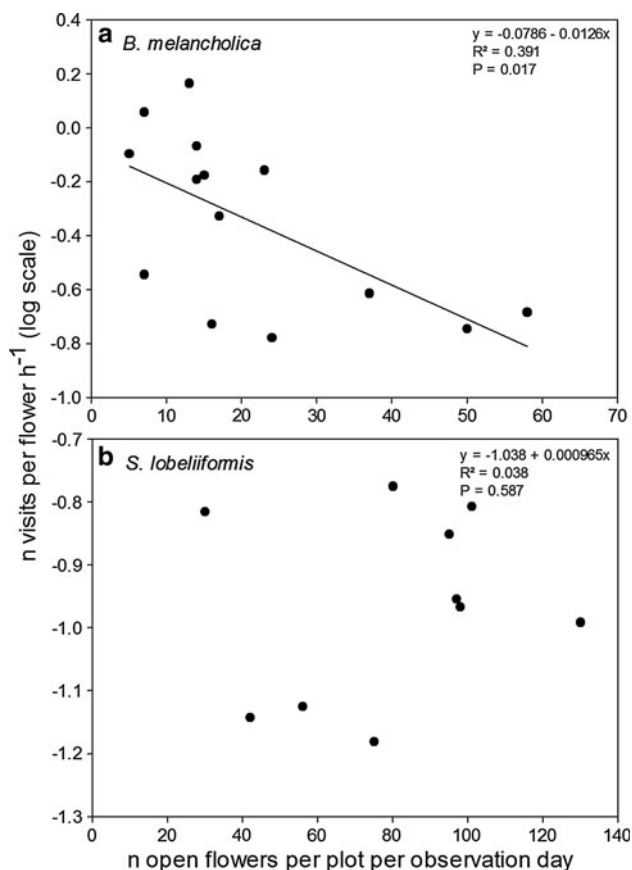
Fig. 1 Linear regression of the number of visits per flower h^{-1} (log-transformed) and the number of open flowers per plot of 2×5 m and observation day (*Besleria melancholica*: 14 days; *Stenostephanus lobeliiformis*: 10 days); of both species (**a** *Besleria melancholica*, **b** *Stenostephanus lobeliiformis*)

(SD 0.04; $n = 80$ h), to one flower of *B. melancholica* 1.5 h^{-1} (SD 1.17; $n = 112$ h) (Table 2). No correlation between flower density and visitor frequency could be found for *S. lobeliiformis*. In contrast, in *B. melancholica* visitor frequency per flower h^{-1} decreased significantly with increasing flower density (Fig. 1).

Breeding systems and female reproductive success

For both species, a significant influence of the treatment, but not the site, on fruit set was found (*S. lobeliiformis*: site: $F = 0.4$, $P = 0.67$; treatment: $F = 8.8$, $P < 0.001$; *B. melancholica*: site: $F = 0.04$, $P = 0.85$; treatment: $F = 5.04$, $P < 0.01$).

Average natural fruit set of unmanipulated control treatments (C) of both species amounted to 0.22 of *B. melancholica* (Fig. 2a) and 0.26 of *S. lobeliiformis* (Fig. 2b). Total fruit loss of all individuals of all treatments to frugivores or herbivores accounted for 20.75 % in *S. lobeliiformis* and 9.68 % in *B. melancholica* (for sample sizes see Table 1). Neither species was autogamous. *B. melancholica* was mostly self-incompatible (ISI = 0.12) and *S. lobeliiformis* was partially self-incompatible (ISI = 0.29). In both species, fruit sets of the outcrossing treatments (OC and HC) were highest among all treatments and differed significantly from fruit sets of the selfing treatments (*B. melancholica*: $T = 51$, $P < 0.001$; Fig. 2a, b; *S. lobeliiformis*: $T = 721$, $P < 0.001$). In *B. melancholica,* no sign of pollen limitation could be found based on the pollination experiments; the PLI was only 1 %. In *S. lobeliiformis*, fruit set of hand crossing was higher than that of natural pollination, suggesting that this species is pollen-limited; the PLI was 22 %.

In the case of *B. melancholica*, seed mass per fruit resulting from the outcrossing treatments was significantly higher than that resulting from the selfing treatments (OC and HC vs. SS and HS, $T = 854$, $P < 0.001$, Fig. 2c). In case of *S. lobeliiformis*, the same pattern could be found, but there was no significant correlation between the different treatments (Fig. 2d).

Germination studies

In *B. melancholica*, seeds from the SS treatment did not germinate, whereas seeds from the HS treatment germinated best out of all treatments (Fig. 3a). In *S. lobeliiformis*, seeds from the treatments AG, SS, and HS did not germinate. Seeds from C showed the highest germination followed by the seeds from HC (Fig. 3b).

Discussion

Flower visitors, attractiveness of the flowers displayed, and nectar sugar composition

We recorded four different flower visitor species for *B. melancholica*. In contrast, *S. lobeliiformis* was visited by 17 different visitor species. This is in accordance with the findings of Frankie (1974) and Augspurger (1980),
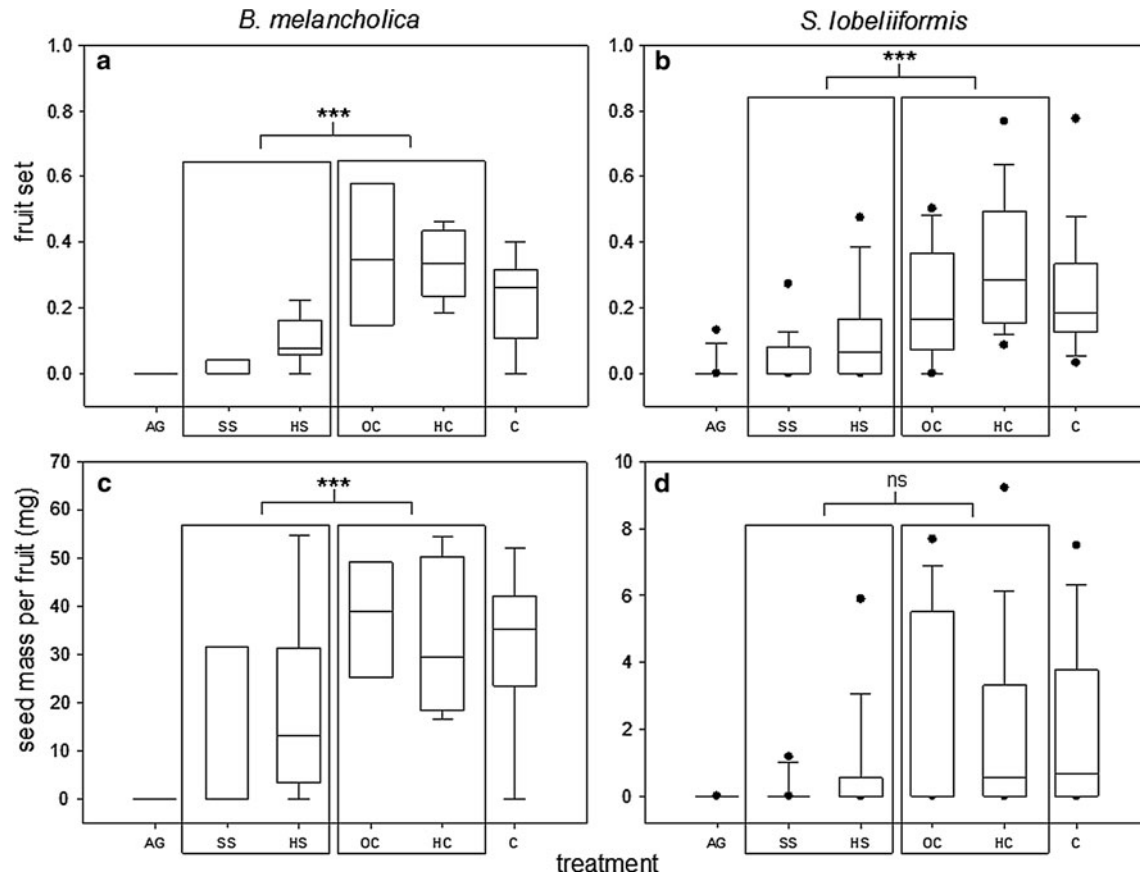
Fig. 2 *Box-plots* showing the average fruit set and average seed mass per fruit of *Besleria melancholica* (**a** fruit set, **c** seed mass) and *Stenostephanus lobeliiformis* (**b** fruit set, **d** seed mass) depending on treatment (*AG* agamospermy, *SS* spontaneous selfing, *HS* hand selfing (geitonogamy), *OC* natural outcrossing, *HC* hand crossing, *C* control). T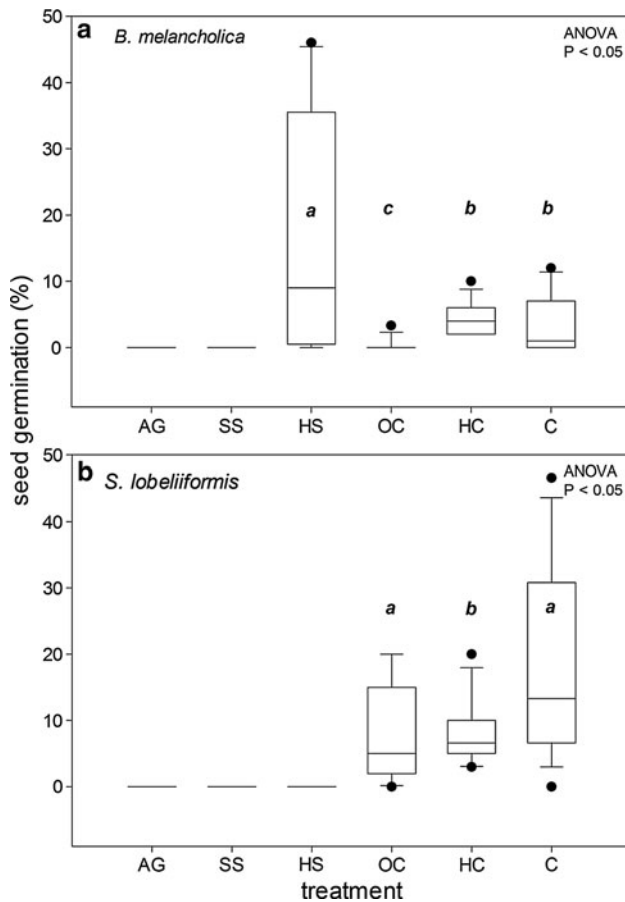he *black line* is the median; the *upper/lower lines* of the boxes are the first and third quartiles; the *vertical lines* are the 5th and 95th percentiles and the *dots* represent outliers of the 5th and 95th percentiles. *Frames around boxes* indicate groups (selfing *SS* and *HS*; outcrossing *OC* and *HC*), which were tested for variance using an ANOVA. Significant differences between the two groups are indicated by *asterisks* (*** $P < 0.001$; *ns* not significant)

who observed pollinators of steady-state (low floral density) flowering species to be low in number and species diversity and that mass-flowering (high floral density) plants attract a large number and high species diversity of "opportunistic" visitors, that means flower visitors with density-dependent foraging behavior.

The flowers of *S. lobeliiformis,* whose floral traits (tubular shape, vivid pink color, no odor) fit to the ornithophilous flowering syndrome, were mostly visited by Hymenoptera (88.8 %), whereas hummingbirds only accounted for 5.5 % of all visits. Plants in general often attract a broader spectrum of visitors than would be predicted from their respective syndromes (Dias da Cruz et al. 2006; Ollerton et al. 2009). Plants with rather specialized flowers are often visited by generalist insects (Ollerton et al. 2003), which also was reported by Robertson et al. (2005) for bird-adapted mistletoe flowers being visited by bees and by Stein and Hensen (2011) for the ornithophilous *Heliconia angusta* visited mostly by stingless bees. Many species of stingless bees, in particular of the genus *Trigona*, are known to rob flowers (Roubik

1982; Renner 1983) by collecting pollen and nectar. We observed these bees being nectar robbers and collecting pollen as well in the two study species. A study of McDade and Kinsman (1980) revealed that species of the genus *Trigona* took pollen from the ornithophilous *Aphelandra golfodulcensis* (Acanthaceae) flowers in Costa Rica without contributing to pollination. On the other hand, stingless bees are considered important pollinators of the native flora in tropical and subtropical parts of the world, and they contribute to the effective pollination of 18 crops and many wild plants (Heard 1999; Slaa et al. 2006). Despite their frequent removal of pollen that otherwise would have been transferred to stigmas by more effective pollinators, species of the genus *Trigona* still have the potential to be secondary pollinators, even if pollination may happen occasionally and rarely during pollen collection. However, the most abundant visitor does not necessarily act as the most efficient pollinator (Fenster et al. 2004; Schmid et al. 2011).

The nectar of both species was found to be sucrose-dominant and diluted, whereas it was poor in the hex-

Fig. 3 *Box-plots* showing the average germination of seeds (**a** *Besleria melancholica*, **b** *Stenostephanus lobeliiformis*) resulting from the different treatments (*AG* agamospermy, *SS* spontaneous selfing, *HS* hand selfing (geitonogamy), *OC* natural outcrossing, *HC* hand crossing, *C* control). Sample sizes are the number of plants, from which seeds were extracted: *Besleria melancholica* 2 (*SS*), 4 (*HS*), 4 (*OC*), 7 (*HC*), 5 (*C*); *Stenostephanus lobeliiformis* 5 (*SS*), 4 (*HS*), 16 (*OC*), 20 (*HC*), 20 (*C*). The *horizontal line in the box* is the median; the *upper/lower lines of the boxes* are the 1st and 3rd quartiles; the *vertical lines* are the 5th and 95th percentiles and the *dots* represent outliers of the 5th and 95th percentiles. *Box-plots* with equal letters do not differ significantly from each other

oses glucose and fructose. Relatively dilute but sucrose-dominant nectar is a common feature in flowers pollinated by hummingbirds, butterflies, and long-tongued bees (Baker and Baker 1990; Baker et al. 1998; Krömer et al. 2008; Stiles and Freeman 1993). Schmidt-Lebuhn et al. (2007) analyzed 70 species of Acanthaceae and found similar low-nectar concentrations (10–15 %) that were sucrose-rich but poor in glucose for other neotropical, hummingbird-pollinated species of Acanthaceae: e.g., *Aphelandra sinclairiana*, *Justicia appendiculata*, and *Sanchezia oblonga*. The flowers of the mass flowering *S. lobeliiformis* were observed secreting a low amount of 5.52 µl on average of nectar per day; in addition, some flowers appeared to be empty, even when bagged. The phenomenon of mass flowering might play an important role, not only in visitor attraction, but as well in floral reward production (Faegri and van der Pijl

1979). It might unburden the individual with regard to attractant and reward production. High flower density might compensate for the minute nectar volumes offered by single flowers (Hobbhahn et al. 2006). It might also allow for the presence of a certain amount of empty flowers, and result in longer foraging bouts in the populations, as found by Brink (1982) and Klinkhamer and van der Lugt (2004).

An increasing number of flowers of *S. lobeliiformis* attracted more Hymenoptera and hummingbirds. A positive correlation between an increasing attractiveness of the plant (e.g., number of open flowers) and the number of floral visitors was also reported by Brody and Mitchell (1997) for the ornithophilous *Ipomopsis aggregata* (Polemoniaceae) and by Stein and Hensen (2011) for *Heliconia angusta* (Heliconiaceae). In contrast, no correlation between floral display size and number of visitors was found for *B. melancholica*.

In general, the visitor frequency to one flower of *B. melancholica* (1.5 visitors per flower h^{-1}) is higher than that to one flower of *S. lobeliiformis* (0.1 visits per flower h^{-1}). These findings support the hypotheses that a large floral display size, despite attracting many visitors, promotes a strong competition of the single flower for visitors (Knight 2003). In *B. melancholica*, visitor frequency decreases with increasing number of flowers displayed per plot, suggesting competition for pollination. In contrast, in *S. lobeliiformis* visitor frequency does not change with increasing floral density. This is probably related to the fact that a higher floral density attracts more visitors, which buffers the frequency.

Breeding system, female reproductive success, and seed germination

Both species are partially or mostly (*B. melancholica*) self-incompatible. The low fruit set resulting from the selfing treatments indicates a strong need for pollinators. Outcrossing treatments (OC, HC) resulted in significantly higher fruit set than the selfing treatments (SS, HS) in both species. Thus, the species are primarily outcrossing. The increased fruit set after hand crossing, compared to natural pollination in *S. lobeliiformis*, suggests pollen limitation. The PLI of *S. lobeliiformis* (22 %) supports the finding. Due to competition of flowers for pollination within the mass flowering population, single flowers might receive insufficient pollen for full seed set. In contrast to our expectations, no pollen limitation could be found in *B. melancholica*. Flowers of *B. melancholica* were visited more frequently than flowers of the dense-flowering *S. lobeliiformis*, thus potentially receiving more pollen. The average observed natural fruit set per control plant accounts for 22 % in *B. melancholica* and for 26 % in *S. lobeliiformis*. Furthermore, the two species have different pollen load requirements for fruit and seed set due to their different numbers of ovules. *S. lobeliiformis* with only four ovules apparently needs fewer pollen grains for fertilization

than *B. melancholica* with thousands of ovules. Wesselingh et al. (1999) studied the pollination of neotropical understory shrubs and found that fruit and seed set in *Palicourea brenesii* with only two ovules per flower were not limited by a shortage of conspecific pollen. In contrast, in *Leandra subseriata*, 20–25 % of the flowers received too few conspecific pollen grains for maximum seed set among 440 ovules per flower.

However, not only pollen quantity but also the quality of pollen received is important for fruit and seed set (Aizen and Harder 2007), which should be subject to further investigations.

Given that predominantly outcrossing species commonly exhibit strong inbreeding depression (Husband and Schemske 1996), self-pollination (e.g., geitonogamy) has frequently a negative impact even on early fitness traits such as seed production and germination (Lienert and Fischer 2004). No seeds of *S. lobeliiformis* from the selfing treatments germinated at all, while the outcrossed and control seeds germinated well, which can be interpreted as a sign of inbreeding depression. In contrast, the seeds of *B. melancholica* resulting from the geitonogamy treatment (HS) germinated best, whereas germination of the outcrossed and control seeds was low. A possible explanation of the low germination might be that the fruits of both species were collected too early. Although the fruits appeared to be mature, the seeds might still have been unripe. Due to the Ornithocheirus fruit dispersal in the case of *B. melancholica*, one had to decide whether to sample the fruits or to take the risk that they would be eaten by birds. A similar problem arose for *S. lobeliiformis*: because mature capsules dehisce explosively, we had to avoid seed loss and thus might have collected the seeds before they were mature.

Based on this limited study, we conclude that the pollination ecology of a species is influenced by a broad set of traits. Flowers in the understories of tropical rainforests, despite their associated floral syndrome, attract a broader spectrum of visitors than might be expected. The floral display size appears to have an important impact on visitor attraction and pollination service. Even a small floral density and rather inconspicuous flowers of a plant species do not necessarily lead to pollen limitation. Field observations and experiments remain essential to investigate the pollination and reproductive ecology of plant species, thus contributing to the knowledge on complex plant–animal-interactions. This might particularly be important for endemic or vulnerable species in a threatened biodiversity hot spot like the Atlantic Coastal rainforest of Brazil.

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