The nectar spur is not only a simple specialization for long-proboscid pollinators

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Introduction

The floral nectar spur is a hollow extension of certain flower parts that often contains tissues producing nectar. The nectar spur is the focal point of many evolutionary and ecological hypotheses, and it was studied by Darwin to understand how natural selection creates adaptive specialized traits (Darwin, 1862). It became an important part of the pollination syndrome hypothesis (van der Pijl, 1960; Faegri & van der Pijl, 1979) and, later, the spur was considered a key innovation that caused the rapid diversification of certain plant phylogenetic lineages (Hodges & Arnold, 1995). Moreover, the nectar spur helped with understanding both individual plant–pollinator interactions (Bartos & Janček, 2014) and the organization of complex plant–pollinator communities (Santamaria & Rodriguez-Girones, 2007; Stang et al., 2007). Recently, the nectar spur has been considered an important trait regulating biological invasions (Vervoort et al., 2011; Chupp et al., 2015). Despite this long research history, many functional aspects of the floral spur remain unknown, and many controversies continue to surround its evolution and function.

Two hypotheses, which are not mutually exclusive, have been proposed regarding the evolution of floral spur length: Darwin’s ‘coevolutionary race’ and the ‘pollinator shift’. The coevolutionary race is based on Darwin’s famous observations of the orchid Angraecum sesquipedale and his prediction that it is probably pollinated by a moth with a proboscis thick enough at the base to pick up pollinia and long enough to reach the nectar in the long spur (Darwin, 1862). This moth was discovered some years later and described as Xanthopan morgani praedicta Rothschild & Jordan, 1903 (Rothschild, 1903), a synonym of Xanthopan morgani Walker, 1856. Darwin’s coevolutionary race hypothesis assumes a gradual increase in the length of a plant’s spur together with the prolongation of the pollinator’s tongue within a species lineage. The driving forces of the coevolutionary race are the advantage for long-proboscid visitors in reaching nectar and the advantage for long-spurred plants in more effective contact between their reproductive organs and the visitor’s body (Nilsson, 1988; Pauw et al., 2009). The pollinator shift hypothesis considers spur length to be the result of an adaptation to a series of unrelated pollinators, each with a longer proboscis than the species before it (Wasserthal, 1997; Whittall & Hodges, 2007). The pollinator shift hypothesis is characterized by rapid changes during a switch to new pollinators by creating reproductive barriers and subsequent speciation. This hypothesis predicts that short-proboscid...
visitors will not be able to reach the nectar and, consequently, will not visit long-spurred flowers and/or that they will not be effective pollinators because they no longer fit the flower’s reproductive organs. Therefore, the plant will become more phenotypically specialized and also more ecologically and functionally specialized (*sensu* Ollerton et al., 2007).

Although these evolutionary hypotheses are widely accepted, empirical data often show patterns that do not fully support them. Community-wide studies show that plant–visitor interactions are much more generalized than previously thought (Waser et al., 1996), and even plants with highly specialized floral traits are visited by diverse assemblages of animals (Waser & Price, 1990; Ollerton, 1996). Flowers with deep corolla tubes often act as nectar reservoirs and, when visitation is prevented, can accumulate high volumes of nectar. Consequently, nectar can reach part way up the tube and, in some cases, even reach the tube entrance (Pleasants & Waser, 1985; Stipcynska, 2003; Martins & Johnson, 2007). Thus, visitors with shorter proboscises may easily reach at least some of the nectar. Furthermore, several previous studies showed that the sugar concentration in the nectar of some spurred (Martins & Johnson, 2007) or tubular (Petit et al., 2011) flowers is not homogenous and suggest that different types of nectar are consumed by different visitors, a fact that probably favors the generalization of pollination systems (Petit et al., 2011). Moreover, other studies revealed diurnal changes of both nectar availability and visitor spectra, indicating possible temporal niche separations (Herrera, 1990). All of the earlier-mentioned studies indicate that the pollination systems of spurred plants need not be highly specialized as predicted by evolutionary hypotheses.

In this study, we focus on *Impatiens burtonii* Hook. f. (Balsaminaceae) and its visitors in Cameroon, West Africa. We studied the pollination effectiveness of individual visitors in relation to traits of the flowers and their visitors. Two components of the effectiveness were studied: the per-visit pollen deposition on the stigma as a quality component and the visitation rate as a quantity component. Based on its floral traits, *Impatiens burtonii* is expected to be pollinated by bees (Grey-Wilson, 1980). We tested three main working hypotheses: (1) nectar accumulates in the spur, where it is exploited by insects of different proboscis, (2) not only long- but also short-proboscid visitors are effective pollinators and (3) over the course of the day, nectar levels will decrease and thus the proportion of long-proboscid pollinators will increase. Moreover, we aimed to discuss how the validity of these hypotheses may change the recent perspectives on evolution and the diversification of spurred flowers.

### Materials and Methods

#### Study site

The study was performed in the Mendong Buo area (6.592699°N, 10.189999°E; 2100–2200 m above sea level), c. 5 km south-east of Big Babanki, in the Bamenda Highlands, North-West Province, Cameroon. This area is a mosaic of extensive pastures, frequently burned forest clearings dominated by *Pteridium aquilinum* (L.) Kuhn, shrubby vegetation along streams, and remnants of species-rich tropical montane forest with the frequent occurrence of *Schefflera abyssinica* (Hoehx. ex A.Rich.) Harms, *Schefflera mannii* (Hook. f.) Harms., *Bersama abyssinica* Fresen., *Syzygium staudtii* (Engl.) Mildbr., *Carapa procera* D.C. and *Ixora foliosa* Hiern. There is a single wet season from March to November, with annual precipitation ranging from 1780 to 2290 mm year⁻¹ (for more details, see Cheek et al., 2000; Reif et al., 2007; Tropek & Konvicka, 2010).

#### Plant species

*Impatiens burtonii* var. *burtonii* Hook. f. (Balsaminaceae) is an erect perennial herb widespread throughout tropical Africa (Grey-Wilson, 1980). It has a disjunct distribution ranging from Cameroon to Kenya and north-western Tanzania in East Africa and is not present in the central Democratic Republic of the Congo (Grey-Wilson, 1980). *I. burtonii* inhabits moist, often densely shaded localities, upland rain forest and forest margins, riverbanks, and the edge of swampy areas, with an altitudinal range of 800–3350 m (Grey-Wilson, 1980). In our study site, it usually grows in the pasture–forest ecotone. Similar to other *Impatiens* species, it is protandrous. In the male phase, united anthers cover the stigma. Later, the flower switches to a female phase in which the anthers fall away and the stigma becomes exposed. It has white or pale pink zygomorphic flowers with a filiform nectar spur that curves downwards. Flowers are produced throughout the entire year (Grey-Wilson, 1980). Grey-Wilson (1980) reported a spur length for *I. burtonii* var. *burtonii* of between 5 and 9 (up to 10) mm.

#### Flower traits

To study diurnal changes in the nectar standing crop, we measured the distance between the nectar level and the spur entrance over 5 d. On each day, we collected 10 flowers at seven different times (06:00, 08:00 and 10:00 h, and 12:00, 14:00, 16:00 and 18:00 h). Because the floral spur is partially transparent, it is usually possible to measure the nectar level without destroying the spur. When we were unsure of the nectar level, we cut off the nectar spur crosswise step-by-step and checked the nectar level using a magnifying glass. If possible, we collected nectar from flowers using 2 µl microcapillary tubes. Because individual flowers did not usually contain sufficient nectar for measurements, we mixed samples from several flowers. We measured the nectar concentration using a Pal-1 (Atago Co., Tokyo, Japan) pocket refractometer. During these measurements, we recorded not only the nectar level but also the total spur length to obtain information about spur lengths in the study area. During our observation of pollinator efficiency in November and December 2012, we observed high amounts of nectar in the flowers of *I. burtonii* in the morning and low amounts in the afternoon. However, the precise data on nectar dynamics were collected later on, in November and December 2016, to support our study.

To estimate flower longevity and the longevities of both male and female sexual phases, in December 2012, we marked 10
flower buds and checked their phase twice per day (at c. 06:00 and 18:00 h).

Observation of visitors

The openings at the fronts of randomly selected flowers were monitored using portable remote video systems on 20 different plants. The portable video system was similar to that described by Kross & Nelson (2011), but we used a VB305-SonyCCD WideLux WDR video camera (Vision Hi-Tech Co. Ltd, Seoul, South Korea) and a RYK-9122 Portable Recorder (Meicheng Co., Taiwan). We recorded visitors in 10 flowers in the male phase in 10 different plants (one flower per plant) from 06:00 to 18:00 h. The same data were recorded for flowers in the female stage. Due to video errors, abscission of the recorded flowers and changes in the flowers’ sexual phase during recording, we did not obtain 120 h of recordings for each sexual stage but instead obtained 92.75 and 97.05 h for 11 female and 11 male flower stages, respectively. In this dataset, two flowers were recorded in both the male and the female stage, as the stage changed during the recording. After fieldwork, we inspected the video-recordings, identified the visitor species and calculated their visiting frequency. We also recorded the feeding behaviors of individual visitors and the numbers of contacts with reproductive organs.

Proboscis insertion

To study trait matching between the floral spurs and proboscis lengths of individual visitors, we cut the spur lengthwise on five flowers, each on a different plant (see Supporting Information Video S1). The spur length of each flower was measured before recording. These flowers were monitored using two portable remote video recording systems. The first recorded the opening of the flower and helped to identify the visitor species. The second camera recorded the spur from the side. The recordings from the second camera were used to measure the depth of insertion of the visitor’s proboscis.

Pollen deposition on stigmas

We individually bagged randomly chosen flower buds in fine mesh to test the quality component of the effectiveness of individual floral visitors. We verified pollen deposition on stigmas of *I. burtonii* by the visitor in just one visit. Flowers were then uncovered in the female phase, when the stigma is exposed, and, after one insect visit, the stigma was collected and stored in 50% ethanol in a 2 ml Eppendorf tube. The visitor was recorded, and pollen grains were subsequently counted using glycine fuchsin jelly under a microscope in the laboratory (Kearns & Inouye, 1993). The contents of each Eppendorf tube were transferred onto a glass slide. After ethanol evaporation, a cube of fuchsin jelly was placed on the glass slide, melted over a flame and covered with a cover slip. Each tube was washed four times. Observations of the flowers were distributed evenly throughout the day (between 06:00 and 18:00 h) to include all possible diurnal visitors and were limited to suitable weather conditions (sunny or partly cloudy). In this single-visit experiment, we observed 42, 21 and 14 pollen depositions by *Rhingia mecyana*, *Melanostoma* sp. and *Apis mellifera*, respectively.

Statistical analyses

Proboscis lengths and deposited pollen grains were compared using permutation ANOVA. To analyse the visitation frequency and time spent on each flower, we used permutation mixed effect models with visitor species identification as the fixed factor and plant (=camera) identification as the random factor. Permutation tests were performed in the PERMANOVA+ program for PRIMER (Anderson et al., 2008). Differences in feeding behaviors of visitors were tested using contingency tables in R software (https://cran.r-project.org/).

Results

We found that three insect species frequently contacted the stigmas of *I. burtonii* flowers and can therefore be considered potential pollinators (Fig. S1). These floral visitors were two dipteran species of the family Syrphidae (also known as hoverflies or flower flies), that is, *R. mecyana* Speiser, 1910 and *Melanostoma* sp., and the honeybee *A. mellifera* Linnaeus, 1758. *Melanostoma* sp. is a new species to science and will be described in a separate taxonomic work. We recorded 88 visits during which the stigma was in contact with the body of *R. mecyana* (mean frequency of contacts = 0.87 h−1), 33 contacts by *A. mellifera* (mean = 0.27 h−1) and 36 contacts by *Melanostoma* sp. (mean = 0.34 h−1) (Fig. S1). *Melanostoma* sp., a member of the subfamily Syrphinae, has a short proboscis (mean = 1.2 mm ± 0.1 SD, n=9) and relatively large labella compared with *R. mecyana* (subfamily Eristalinae), which has one of the longest proboscises within the family (mean = 7.67 mm ± 1.25 SD, n=11) and a relatively smaller labellum. The mean tongue length of *A. mellifera* was 4.47 mm (± 0.86 SD, n=9). These three insects differed markedly in the depths to which they were able to insert their proboscises into the flower spur (perm. ANOVA; \(F_{2,23} = 46.53; \ P < 0.01\)). Whereas *R. mecyana* reached 81% (7.6 mm) of the nectar spur length on average and was sometimes able to reach the end of the spur, *A. mellifera* reached on average < 50% (4.2 mm) of the spur length, and *Melanostoma* sp. reached only c. 14% (1.3 mm) (Fig. 1). *R. mecyana* and *A. mellifera* fed mainly on nectar, whereas the short-tongued *Melanostoma* sp. fed frequently on pollen (\(\chi^2 = 46.26; \ P < 0.01\); Fig. 2a). Regarding pollen, both species of hoverflies ate pollen from anthers, whereas *A. mellifera* collected pollen into pollen baskets. *Melanostoma* sp. also spent much longer on each flower per visit than the other two visitor species (perm. mixed-effect model; \(F_{2,197} = 12.47; \ P < 0.01\); Fig. 2b).

The two components of pollinator effectiveness, visitation rate and pollen deposition per visit, differed among flower visitors. Visitation frequencies were recorded for 20 flowers. Two of these flowers were observed in both the male and the female phases because the phase changed during the observation. The most frequent visitor to flowers in the female phase was *R. mecyana*, followed by *Melanostoma* sp. and *A. mellifera* (perm. mixed-effect model; \(F_{2,197} = 12.47; \ P < 0.01\); Fig. 2b).
model; $F_{2,20} = 3.99; P < 0.05$; Fig. 3a); however, the rates of visitation of the three species to flowers in the male stage were similar (perm. mixed-effect model; $F_{2,20} = 0.96; P = 0.42$; Fig. 3b). In a separate single-visit experiment, the highest number of pollen grains was deposited by the honeybee, followed by R. mecyana and Melanostoma sp. (perm. ANOVA; $F_{2,74} = 6.06; P < 0.01$; Fig. 3c). However, post hoc comparison of A. mellifera and R. mecyana showed no significant difference ($P = 0.16$; Fig. 3c).

We found only a few heterospecific pollen grains on I. burtonii. There is another common Impatiens species (I. sakeriana) in the studied area, and the pollen grains of these two plant species cannot be distinguished. However, we do not expect any bias in the data, since I. sakeriana is pollinated by birds and is not visited by insects (Janeček et al., 2011, 2015; Padyšáková & Janeček, 2016). Pollinator effectiveness, calculated as the rate of pollen deposition ($= \text{no. of pollen grains deposited on virgin stigmas} \times \text{visitation frequency on female-stage flowers}$), was highest for R. mecyana (75.7 pollen grains h$^{-1}$), followed by A. mellifera (36.3 h$^{-1}$) and Melanostoma sp. (15.2 h$^{-1}$).

There was a pattern in diurnal visitation to both the female and the male flower phases. In the early morning, flowers were visited mainly by the short-proboscid Melanostoma sp. Subsequently, we recorded the peak of activity of the mid-tongued A. mellifera before noon, and, in the afternoon, we observed frequent visits by the long-proboscid R. mecyana (Figs 4a, S2). Our video recordings of cut spurs also revealed that whereas A. mellifera and R. mecyana searched for nectar hidden in the long tube, Melanostoma sp. licked the nectar remnants from the corolla walls near the spur entrance (Video S1).

The distance from the spur entrance to the nectar level changed accordingly throughout the day (Fig. 4b). In the morning, bees were able to reach the nectar in 25–50% of the flowers, but in the afternoon, in the vast majority of flowers, the nectar was available only for R. mecyana. The short-proboscid Melanostoma sp. rarely reached the nectar. We recorded a distance to the nectar level that would be accessible to the Melanostoma sp. proboscis (up to 2 mm) for only 26 of 352 (i.e. 7.6%) measured flowers. Fifteen of these flowers were collected at 06:00 or 08:00 h, when Melanostoma sp. was most active. Mean nectar concentration was 35.6% (SD = 7.3; $n = 23$), and there were no diurnal changes. Total flower longevity was $4.6 \pm 0.4$ d. Durations of the male and female phases were $3.9 \pm 0.5$ and $0.7 \pm 0.5$ d, respectively. In our study area, we reported a mean nectar spur length of 8.97 mm ($\pm 0.9$ SD, $n = 352$, range 6–12 mm, Fig. S3).

**Discussion**

In the present study, we supported our working hypotheses that (1) nectar accumulates in the spur, where it is exploited by insects of different proboscis lengths, (2) I. burtonii is effectively pollinated by both short- and long-proboscid pollinators and (3) over
the course of the day, the nectar level decreases, and the proportion of visits of long-proboscid pollinators increases. These empirical results challenge some aspects of the two widely accepted evolutionary hypotheses, that is, Darwin’s coevolutionary race hypothesis (Darwin, 1862) and the pollinator shift hypothesis (Wasserthal, 1997; Whittall & Hodges, 2007). Moreover, these results may contribute to the discussions of the diversification of many plant phylogenetic lineages.

We found that *I. burtonii* is not pollinated only by bees as expected (Grey-Wilson, 1980) but instead that the pollination system is more complex, with two hoverflies and the honeybee as the main pollinators, which differentially utilize the resources offered by *I. burtonii*. The long-proboscid hoverfly *R. mecyana* deposited on stigmas the highest number of pollen grains per time unit. However, the highest effectiveness of *R. mecyana* was due to its high visitation frequency, whereas the highest pollen deposition per visit was observed for *A. mellifera*. Our results support other studies showing the lack of a positive correlation between quality (e.g. per-visit pollen deposition) and quantity (e.g. visitation rate) components of pollinator effectiveness (Pettersson, 1991; Gómez & Zamora, 1999; Mayfield *et al.*, 2001). Nonetheless, we did not measure the male components of fitness, that is, the relationship between pollen grains removed from the anthers and pollen grains deposited on the stigmas. Such an approach might well change our perspective on the
effectiveness of *Melanostoma* sp., which feeds on pollen to a high degree and may show low relative pollen transport effectiveness.

We predict that the main factors maintaining ecological and functional generalization in the studied pollination systems are spatial and/or temporal niche separation. Our study, similarly to other previous studies, shows that main foraging activity of *Melanostoma* sp. occurs early in the morning (Morse, 1981; Ssymank, 2001). In the morning, *Melanostoma* sp. can feed not only on pollen and nectar remnants near the spur entrance but can also reach nectar levels in some filled flowers. Before noon, there is sufficient nectar to be used by the honeybee, but the afternoon feeding is restricted to the long-proboscid *R. mecyana*. The hoverfly *R. mecyana* was the only visitor able to reach the end of the spur. Similar results were reported by Laverty & Plowright (1985) in the pollination system of *Impatiens capensis* Meerb. (as *Impatiens biflora* Walter, jun. syn.). *I. capensis* is frequently visited by bumblebees with shorter tongues in the early morning, whereas long-tongued bumble bees dominate during the day. The authors also demonstrated spatial niche separation in the presence of hummingbirds, in which hummingbirds foraged on the outermost exposed flowers of *I. capensis*, whereas bumblebees visited the innermost. Furthermore, different mechanisms of nectar consumption can enable visitors to use different feeding niches and lead to higher generalization of the *I. burtonii* pollination system. Syrphid mouthparts form a tubular sucking organ (proboscis and labella) with pumps operated by muscular contraction (Gilbert, 1981). By contrast, the mouthparts of the honeybee, a tongue (glossa) with erectable hairs (Snodgrass, 1956; Zhu et al., 2016), capture nectar on the outer surface (Kim & Bush, 2012). Moreover, *Melanostoma* sp., the visitor with the shortest proboscis, sucks nectar remnants from the spur walls (Video S1).

Both Darwin’s coevolutionary race hypothesis (Darwin, 1862) and the pollinator shift hypothesis (Wasserthal, 1997; Whittall & Hodges, 2007) predict that during evolution of long-spurred flowers, the short-proboscid pollinators are excluded from the pollination system, and the long-proboscid pollinators are expected to be the only ones producing selection pressure on flower traits. By contrast, our results show that even visitors with shorter proboscises can be effective pollinators and that the possible selection pressures on flower traits can therefore be much more diverse.

We believe that unless there is a better understanding of the roles of individual plant and pollinator traits and related selection pressures, it will be impossible to understand the functioning and evolution of individual pollination systems. For example, spur and proboscis lengths may be influential in some pollination systems on the quantity component of pollinator effectiveness (i.e. on visitation frequency), whereas other traits may be relevant for the quality component of pollinator effectiveness (i.e. pollen deposition per visit). In the case of Darwin’s system with *A. sesquipedale* and *X. morgani*, these quality-related traits are the arrangement of pollinia and the thickness of the proboscis at the base, respectively. Only a thick (not necessarily long) proboscis can effectively touch and remove pollinia. Mayfield et al. (2001) suggested that the hairy bodies of bumblebees may ensure excellent contact with sexual organs of *Ipomopsis aggregata* (Pursh) V.E. Grant (Polemoniaceae), regardless of the fact that the long corolla seems to be an adaptation to hummingbird pollination. In the case of our study, the insect must be large enough to touch the anthers and stigma when sitting on the lower petals during feeding (see Fig. 1). It is also possible that the hairs on the honeybee thorax can increase its effectiveness, similar to the suggestions mentioned by Mayfield et al. (2001). Regarding flower spur length, we assume that *R. mecyana* creates the strongest selection pressure on this flower trait; this would support the most effective pollinator principle hypothesis, which assumes that the flower traits are determined by the most effective pollinator (Stebbins, 1970). However, as suggested by Aigner (2001), we accept that other traits can also be shaped by less-effective pollinators in situations in which these adaptations require little loss in the fitness contribution of the most effective pollinator. Such adaptations in spurred flowers may include, for example, a high production of...
nectar. Then, if the competition pressure by long-tongued visitors is weak, nectar is also available for pollinators with shorter probosces.

Our observations and results support a quite different scenario for the pollination system of spurred flowers if the long-proboscid pollinators disappear, in contrast to the coevolutionary race and pollinator shift hypotheses. We suggest that in complex plant–pollinator communities, plants may easily switch to pollination by short-tongued pollinators. This scenario is consistent with the well-studied pollination system of *I. aggregata*. This plant has a bird pollination syndrome, and it is usually pollinated by hummingbirds. Nevertheless, in years when the visits of hummingbirds are relatively infrequent, the nectar surface is closer to the flower tube entrance, and bumblebees, which start to be more frequent visitors, are able to deliver higher pollen loads to the virgin stigmas (Pleasants & Waser, 1985; Mayfield *et al.*, 2001). In general terms, our observations are consistent with increasing evidence that pollination systems are more generalized than previously thought (Waser *et al.*, 1996).

The possibility of an evolutionary shift to the short-proboscid pollinators should be corroborated in further studies. These studies should include the elimination of long-proboscid pollinators from the pollination systems or the reduction of competitive pressure (e.g. experimental maintenance of the nectar volume in the flowers). The experimental demonstration of the shift may have significant implications regarding the hypotheses on the diversification of many phylogenetic plant lineages. Under this scenario: (1) spurred plants would be much more resistant to the changes in pollinator communities; (2) selection pressures in individual populations would be much more diverse; and (3) this geographical coevolutionary mosaic (in the sense of Thompson’s theories; Thompson, 2005) would lead to rapid diversification. Based on recent global changes, this scenario presents both bad and good news. Although it supports the robustness of plant–pollinator interactions and reduces the probability of the extinction of individual species, it also predicts much greater ease of invasion of alien species into new areas. Future studies should also address selection pressures in individual *I. burtonii* populations and possible seasonal changes. Comparisons among *I. burtonii* var. *burtonii* and East African varieties (var. *wittei* and var. *angusticalcarata*), which differ in spur lengths (Grey-Wilson, 1980), are also necessary.

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**Author contributions**

A.V., E.P., M.B., P.J. and Š.J. performed the laboratory and field work. Š.J. analysed the data. X.M. identified the dipteran visitors. A.V., E.P., M.B., X.M., P.J. and Š.J. wrote the manuscript.

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**Supporting Information**

Additional Supporting Information may be found online in the Supporting Information tab for this article:

**Fig. S1** Most common visitors of *Impatiens burtonii*.

**Fig. S2** Diurnal changes in visitation frequencies to male flowers of *Impatiens burtonii*.

**Fig. S3** Histogram of nectar spur lengths at the study site.

**Video S1** Frequent visitors of *Impatiens burtonii*.

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