

RESEARCH PAPER

Beyond the pollination syndrome: nectar ecology and the role of diurnal and nocturnal pollinators in the reproductive success of *Inga sessilis* (Fabaceae)

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ABSTRACT

Inga species present brush-type flower morphology allowing them to be visited by distinct groups of pollinators. Nectar features in relation to the main pollinators have seldom been studied in this genus. To test the hypothesis of floral adaptation to both diurnal and nocturnal pollinators, we studied the pollination ecology of *Inga sessilis*, with emphasis on the nectar secretion patterns, effects of sequential removals on nectar production, sugar composition and the role of diurnal and nocturnal pollinators in its reproductive success. *Inga sessilis* is self-incompatible and pollinated by hummingbirds, hawkmoths and bats. Fruit set under natural conditions is very low despite the fact that most stigmas receive polyads with sufficient pollen to fertilise all ovules in a flower. Nectar secretion starts in the bud stage and flowers continually secreting nectar for a period of 8 h. Flowers actively reabsorbed the nectar a few hours before senescence. Sugar production increased after nectar removal, especially when flowers were drained during the night. Nectar sugar composition changed over flower life span, from sucrose-dominant (just after flower opening, when hummingbirds were the main visitors) to hexose-rich (throughout the night, when bats and hawkmoths were the main visitors). Diurnal pollinators contributed less than nocturnal ones to fruit production, but the former were more constant and reliable visitors through time. Our results indicate *I. sessilis* has floral adaptations, beyond the morphology, that encompass both diurnal and nocturnal pollinator requirements, suggesting a complementary and mixed pollination system.

INTRODUCTION

Generalist pollination systems are more common and widely distributed than pair-wise species interactions (Waser *et al.* 1996; Fenster *et al.* 2004; Freitas & Sazima 2006), and can be viewed as a successful evolutionary tendency within some plant groups (Torres & Galetto 2002). Floral adaptations to a pollinator group depend on its spatiotemporal reliability by means of the visitation frequency and pollination effectiveness (Stebbins 1970; Artz *et al.* 2010; Burkle & Alarcón 2011). Since flower morphology is moulded by plant–pollinator interactions, mixed or generalised pollination systems are favoured and eventually fixed in a plant population, when plants are effectively pollinated by different groups of pollinators (Waser *et al.* 1996; Fleming *et al.* 2001; Brunet & Holmquist 2009; Sahli & Conner 2011). However, to be effective, pollination depends on the matching between particular characteristics of flowers (*e.g.* morphology, anthesis duration, scent and reward production) and pollinator morphology, as well as behaviour (Armbruster *et al.* 2004, 2009).

Pollination syndromes cannot be accurately predicted for most flowering plants solely through floral morphology observations (Waser *et al.* 1996; Ollerton *et al.* 2009). Flower-

ing phenology, timing of flower opening, anthesis duration and nectar chemical compounds and secretion patterns are also directly related to the pollination system and pollinator activity on flowers (Southwick *et al.* 1981; Baker & Baker 1983; Pleasants 1983; Galetto & Bernardello 2005; Bobrowiec & Oliveira 2012). As such, the traditional concept of pollination syndromes cannot be used precisely to define the most common or effective pollinator (Freitas & Sazima 2006; Ollerton *et al.* 2009), only as general hypotheses to design studies on pollination ecology.

Some species are susceptible to flower visitor activity both during the day and at night, especially those with flowers that remain opened and functional for long periods of time. In those species, the definition of the most efficient pollinator group depends not only on quantifying the relative frequency of each group, but also on exclusion experiments of day or night visitors, aiming to quantify the relative contribution of each pollinator group to the plant reproductive success (Miyake & Yahara 1998, 1999; Fleming *et al.* 2001; Young 2002; Wolff *et al.* 2003; Giménez-Benavides *et al.* 2007; Muchhala *et al.* 2009; Maruyama *et al.* 2010; Walter 2010; Ortega-Baes *et al.* 2011). Night- and day-pollinated plants generally involve complementary pollination systems, in which pollen

transfer among flowers is maximised when flowers remain open, taking advantage of the activity of both diurnal and nocturnal pollinators (Jennersten & Morse 1991; Sazima *et al.* 1994; Miyake & Yahara 1998, 1999; Maruyama *et al.* 2010).

Nectar is the main reward in flowering plants and is consumed by a huge variety of animals (Proctor *et al.* 1996). Composed mainly of sugars, nectar can also include amino acids, proteins, lipids and many other substances in its composition. Especially for nectar sugar composition, there are some general trends associating nectar to the pollinator guild. Flowers visited mainly by hummingbirds and sphingids, for example, tend to present sucrose predominant nectars, whereas those visited by bats are hexose predominant (*e.g.* Baker & Baker 1983; Von Helversen 1993; Koptur 1994; Galetto & Bernardello 2003; Chalcoff *et al.* 2006). Also other nectar features, such as secretion rhythms and production pattern, may be strongly influenced by interaction with pollinators (Bobrowiec & Oliveira 2012; but see Galetto & Bernardello 2005 and references therein). Since natural selection favours plant adaptation to the 'most effective pollinator' (*sensu* Stebbins 1970), patterns of floral reward production, *e.g.* nectar, may correspond to the activity period of the main pollinators (Heinrich 1975). Hence, in night- and day-pollinated plants, if both groups of flower visitors act as effective pollinators, we should expect nectar characteristics (*e.g.* secretion pattern and sugar composition) congruent to the requirements of both pollinator groups.

Among plants pollinated by day and night visitors are many species of *Inga* Willd. (Fabaceae), a common tree genus in Neotropical regions (Pennington 1997). The genus includes about 300 exclusively Neotropical species, which possess brush-type flowers with reduced calyx and corolla parts, gynoecium with one single carpel, usually unilocular, and one pistil that equals or exceeds the length of the stamens. The androecium acts as the main flower display to visitors, consisting of a set of numerous stamens fused at the base, forming a tube where the nectar is located and offered as the main reward to flower visitors (Koptur 1983; Pennington 1997). This arrangement of the floral morphology allows the nectar to be accessed by a wide spectrum of visitors, ranging from small bees and moths to large hawkmoths, as well as vertebrates, such as hummingbirds and bats (Koptur 1983, 1984; Pennington 1997). Thus, the pollination of a single *Inga* species may involve many distinct groups of visitors (Koptur 1983, 1984; Pennington 1997).

Therefore, *Inga* species can be used as a good model to test general hypotheses of flower adaptations (*e.g.* nectar secretion patterns, sugar composition) to different groups of pollinators, also to investigate the role of diurnal and nocturnal pollinators to the reproductive success of species with different groups of pollinators. In this sense, we hypothesise an adjustment between nectar sugar composition and secretion dynamics through flower anthesis with the activity and requirements of diurnal and nocturnal pollinators, as well as a correspondence with their role in the reproductive success of *Inga sessilis* (Vell.) Mart. In order to evaluate this hypothesis we obtained data on (i) the activity of nectar secretion during the flower life span; (ii) the effect of sequential nectar removal on its total production; (iii) the nectar sugar composition; and (iv) the main pollinators and effectiveness of diurnal and nocturnal flower visitors on fruit set.

MATERIAL AND METHODS

Study site and species characterisation

This study was conducted between 2008 and 2011 in a highland Atlantic rain forest area (montane ombrophilous dense forest) in the Núcleo Santa Virginia (NSV) (23°17'–23°24' S, 45°03'–45°11' W) of the Serra do Mar State Park (SMSP), Brazil. The total area encompassed by the reserve is approximately 5000 ha and is situated between the municipalities of São Luiz do Paraitinga, Cunha, and Ubatuba (Veloso *et al.* 1991). The altitude varies from 850 to 1200 m a.s.l., and the climate is tropical temperate (Cwa, according to Köppen 1948), with average annual precipitation above 2000 mm and never falling below 60 mm during the driest months (Setzer 1966).

Inga sessilis is a tree species that can reach up to 18 m in height, and its distribution is associated with rain forests in southeast Brazil. In the Atlantic rain forest, *I. sessilis* occurs mainly in montane forests, inside the forest or associated with secondary vegetation areas, as well as at roadsides (Pennington 1997). At NSV, the species is commonly observed on the margins of the state highway Oswaldo Cruz (SP-125), which transverses the NSV and where plants are lower in height, ranging from 4 to 10 m.

Phenology and floral biology

Floral phenology was observed in 20 plants using Fournier's (1974) semi-quantitative method. Flowering intensity was quantified by attributing numerical grades, which assigned the relative percentage of the number of flowers produced per plant. The grades varied between 0 and 3, with 0 indicating the absence of flowers, and 1, 2 and 3 indicating presence in the following percentage intervals: 1–25%, 26–75% and 76–100% of branches on one observed plant. Flowering pattern was then classified following Newstrom *et al.* (1994).

Floral anthesis was observed in 200 marked flowers in ten plants (20 flowers per plant). Flowers were followed from opening until senescence, characterised by the wilting of stamens. Stigmatic receptivity was tested using an aqueous solution of 20% hydrogen peroxide (Kearns & Inouye 1993), and the emission of floral odours was recorded in the field from 20 (five plants) fully opened flowers, which were sealed in glass jars from 10 to 15 min and then qualitative floral odour was described (Kearns & Inouye 1993).

To determine the operative flower length, considered as distance between the nectary and the anthers/stigma, ten flowers from five plants were measured with digital calipers (error 0.01 mm). We quantified the number of ovules per ovary in 32 flowers from eight plants. The number of pollen grains per polyad and pollen viability was recorded in 40 polyads from four plants, one flower per plant, collected during the pre-anthesis period. Five anthers per flower were dissected under a stereomicroscope to quantify the number of polyads per anther and the number of pollen grains per polyad. Two polyads per anther were stained with 2% acetic carmine and a gentle pressure was applied on the slide cover to isolate the pollen grains. The pollen viability was estimated from the number of fully stained pollen grains in each polyad. Pollen/ovule ratio (P/O) was determined by multiplying

the number of pollen grains per polyad by the number of polyads per anther and then by the average anthers per flower. The number of anthers was quantified in 20 flowers from 20 plants, by counting the number of stamens per flower. To investigate the potential capacity of one polyad to fertilise all ovules in a single ovary, we calculated the ratio of the number of pollen grains per polyad by the mean number of ovules per ovary (PP/O).

Nectar: secretion pattern, removal effects and sugar composition

Nectar secretion dynamics, floral response to repeated nectar removals and sugar composition analyses were performed according to Torres & Galetto (1998) and Galetto & Bernardello (2005). Nectar volume was measured using a graduated microliter syringe (Hamilton, NV, USA) and sugar concentration (percentage sucrose, mass/mass) was measured with a pocket refractometer (0–50%; Atago, Tokyo, Japan). The amount of sugar in the nectar was quantified and expressed in milligrams following Galetto & Bernardello (2005). Nectar secretion pattern was determined using nine plants in which 35 flowers (three to five flowers per plant) were isolated in nylon mesh bags during the bud stage. Nectar dynamics were obtained during a 24-h period (17:00 to 17:00 h) at 4-h intervals, with nectar measures made from flower opening until senescence. In total, the dynamics of nectar throughout anthesis was analysed in seven flower sets of five flowers each. The nectar was drained and measured once in each flower set, allowing the nectar to accumulate until the next measurement in an untouched new flower set. Each flower set consisted of flowers from different individuals in order to cover inter-plant variations and determine nectar dynamics (according to Galetto & Bernardello 2005). The different phases of the nectar dynamics linked to the accumulated nectar in the different flower sets (*i.e.* active nectar secretion, cessation and resorption) were inferred from the plotted values of these data. The net nectar production rate per hour (NNPR) was calculated as the ratio of the amount of sugar secreted (in mg) in each interval by the number of hours of the secretion period. The net nectar resorption rate (NNRR) was similarly calculated as the ratio of the amount of nectar resorbed (in mg) by the number of hours of the resorption period (Torres & Galetto 1998; Galetto & Bernardello 2005).

Floral response to successive nectar removals was evaluated in an experimental design simulating the activity of flower visitors (*i.e.* multiple visits to the same flowers) over the course of anthesis. We compared total accumulated nectar between 'visited' (*i.e.* nectar experimentally drained) and unvisited flowers (control) in seven sessions at 4-h intervals during the flower lifespan in seven flower sets. A new untouched flower set was included in each subsequent removal session. Thus, in the first set of flowers (set 7), the nectar was drained seven times, the second (set 6) six times, and so on until set 1 (see details in Galetto & Bernardello 2005). In this last group (control), nectar was drained only once allowing its accumulation until the end of flower anthesis, marked by the wilting of stamens.

To analyse the nectar sugar composition, nectar samples were collected in the field and stored on Whatman No. 1

chromatography paper (Galetto & Bernardello 2005). These samples were processed with gas-liquid chromatography (GLC) to determine the types of sugar in nectar and their respective proportions (Galetto & Bernardello 2005). The sucrose/hexose ratio was calculated following Baker & Baker (1983), as: sucrose/(glucose + fructose), in which values below 0.1 indicate hexose-rich nectar, and ≥ 1.0 are sucrose-rich nectar. Intermediate values between 0.1 and 0.499 indicate nectar hexose-dominant and ≥ 0.5 as sucrose-dominant nectar. Samples were taken from non-visited flowers during three periods of anthesis: first between 17:00 and 18:00 h, second between 21:00 and 22:00 h and finally in the morning, between 07:00 and 08:00 h. The same set of flowers was used to obtain nectar samples for GLC analyses.

Pollinators and compatibility system

Floral visitors and pollinators were determined using focal observations in groups of 40–60 flowers per plant, a total of 40 h. In February 2009, the frequency of floral visitors was quantified for 2 h, between 22:00 and 00:00 h. In February 2011 the observations were conducted in sessions of 15 min every 30 min between 16:00 and 01:00 h. The remaining observations were only qualitative (without quantifying the frequency of floral visitors), conducted between 06:00 and 17:00 h in 20 plants. Observations of nocturnal visitors were performed using night-vision goggles, and floral visitors were photographed for identification. In order to record richness and abundance of the potential pollinators, hawkmoths were collected each month in light traps, throughout a period of 1 year, and the pollen loads were analysed to identify those species that carried *I. sessilis* polyads.

The pollination efficiency was estimated by the proportion of polyads deposited on stigmatic surfaces of flowers exposed to visitors during their lifespan. Polyads deposition was quantified observing pistils under fluorescence microscopy for analysis of pollen tube growth (Martin 1959). For this purpose, 40 flowers (3–4 flowers per plant) were collected from 12 plants in May 2011.

To evaluate the compatibility system, experiments with controlled pollination were conducted. Flower buds in pre-anthesis were previously isolated with nylon mesh bags to exclude floral visitors. For these experiments, we used 12 individuals, at least 0.5 km apart, to perform the following pollination treatments: cross-pollination (flowers were emasculated and hand-pollinated with polyads from other plants); self-pollination (flowers were hand-pollinated using polyads of the same plant); spontaneous self-pollination (unmanipulated flowers were kept bagged); emasculation (pre-anthesis flowers were emasculated to verify fruit formation by apomixis); and a control (flowers were marked but not bagged and remained exposed to pollinators). The index of self-incompatibility (ISI *sensu* Bullock 1985) was calculated as the ratio between the number of fruits formed by self-pollination/cross-pollination. The upper limit adopted to consider a species as self-incompatible was 0.25 (according to Oliveira & Gibbs 2000).

Pistils from 20 self- and 20 cross-pollinated flowers were collected 24 h after pollination and fixed in a solution of formalin, acetic acid and ethylic alcohol 50% (FAA 50%) for 24 h, and subsequently stored in ethylic alcohol 70%. The

pistils were stained with aniline blue for pollen tube growth observation under a fluorescence microscope (Martin 1959).

Efficiency of diurnal versus nocturnal pollinators

In order to assess the effectiveness of the day and night pollinators, exclusion experiments were conducted. To exclude diurnal visitors, flowers were isolated with nylon mesh bags just before dawn (around 04:30–05:30 h) until late afternoon on the same day, and to exclude nocturnal visitors, flowers were bagged at dusk (around 17:00–18:00 h) until sunrise (around 05:30–06:00 h), when they were exposed to daytime visitors. Pollinator exclusion experiments were conducted in March 2010 and January 2011. During the first year, seven plants were used and 87 and 89 flowers were isolated during the day and night, respectively. In the second year, ten plants were used, in which 246 flowers were used per treatment (day and night). In total, 668 flowers were used: 333 for the exclusion of diurnal visitors and 335 for exclusion of nocturnal visitors. The fruit set was evaluated 35–60 days after pollinator exclusion treatments.

Statistical analyses

To analyse the effect of experimentally drained nectar (*i.e.* removals by floral visitors) to the total amount (milligrams) of sugar produced by each set of flowers, we performed a one-way analysis of variance (ANOVA) to evaluate the differences in the mg sugars among sets of flowers, using a Tukey *post-hoc* test for multiple comparisons among pairs of means (Zar 2010). These differences were evaluated comparing set of flowers before the resorption period (until set 2). Hence, set 1 was not included in the statistical analysis, since the effects of successive nectar removal on its total production are mixed with resorption effects. The difference in the total number of fruits produced during both years by diurnal and nocturnal pollinators was assessed with a Chi-square test (χ^2). We did not analyse each year separately as the number of fruits was too small for statistical analysis.

RESULTS

Phenology and floral biology

Inga sessilis is a mass-flowering species and blooms throughout the year, displaying a continual flowering pattern (*sensu* Newstrom *et al.* 1994). However, two flowering peaks were more evident (one from December to February and a shorter during May–June), in which the majority of plants were

flowering (Fig. 1). The species present brush-type hermaphroditic flowers arranged in axillary racemes. Flowers are white, have a reduced perianth, a single pistil and numerous stamens (184.20 ± 28.05) (Fig. 2A). The distance between the nectary and anthers/stigma (flower length) is 8.51 ± 0.38 cm. Anthesis lasts 24 h, and flower opening begins around 11:00–13:00 h with a slow expansion of the pistil and stamens. Flowers only become fully open late in the afternoon, around 17:00 h, at which point the pistil and stamens were totally expanded, anthers were dehiscent exposing the polyads, and concomitantly the stigma was receptive, as characterised by the shiny appearance of its surface. Flower scent was slightly sweet throughout its life span. Flower senescence began on the following day and was characterised by wilting of stamens, but the pistil remained erect, not wilting until the next day.

Flowers presented 24.19 ± 3.06 ovules per ovary, and each anther contained 12 ± 0.0 polyads, each composed of 32 ± 0.0 pollen grains with 98% viability. Eleven (27.5%) of 40 polyads showed non-viable pollen. Considering the mean number of stamens per flower (184.20 ± 28.05), the total number of pollen grains in each flower was over 70,000; thus, the P/O ratio was approximately 2944 and PP/O was 1.33.

Nectar: secretion pattern, removal effects and chemical composition

Nectar secretion started during pre-anthesis, and newly opened flowers contained around 50–100 μ l, with 6% sugar concentration (Fig. 3A and B). Activity of nectar secretion in bagged flowers lasted 8 h, with a NNPR of $4.13 \text{ mg}\cdot\text{h}^{-1}$. After 01:00 h, nectar volume, concentration and amount of sugars remained constant over the following hours (Fig. 3), hence this period was interpreted as a cessation of nectar secretion. After which a reduction in the nectar standing crop of the bagged flowers was detected. During this stage, there was a constant decrease of nectar volume and amount of solutes (Fig. 3C), and flowers began to wilt. This period was interpreted as one of active nectar resorption, in which nectar recovery by the flower started a few hours before flower senescence, with a NNRR of $6.33 \text{ mg}\cdot\text{h}^{-1}$.

Experimentally drained flowers increased nectar production up to twofold in the total amount of sugar (TAS) per flower (Table 1). ANOVA comparisons showed significant differences ($F_{5,23} = 4.092$, $P = 0.0085$), between the control (set 2, TAS = 26.27 ± 22.99 mg) and set 4 (TAS = 50.63 ± 10.43 mg) and set 5 (TAS = 56.53 ± 7.28 mg; Table 1). Nectar sugar composition changed over flower life span. In newly

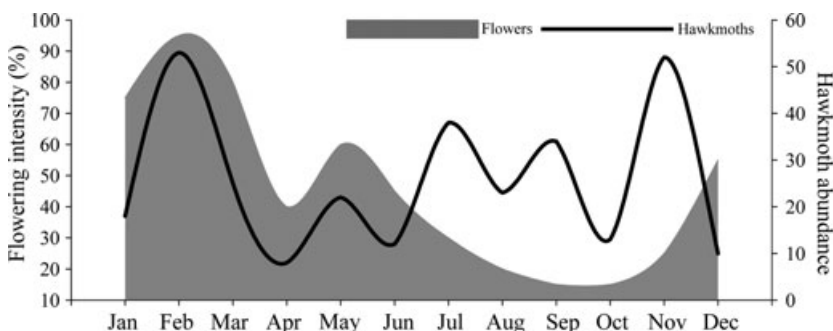


Fig. 1. Flowering phenology of *Inga sessilis* and abundance of its hawkmoths in a montane ombrophilous dense forest at the Núcleo Santa Virginia, Serra do Mar State Park–SP.

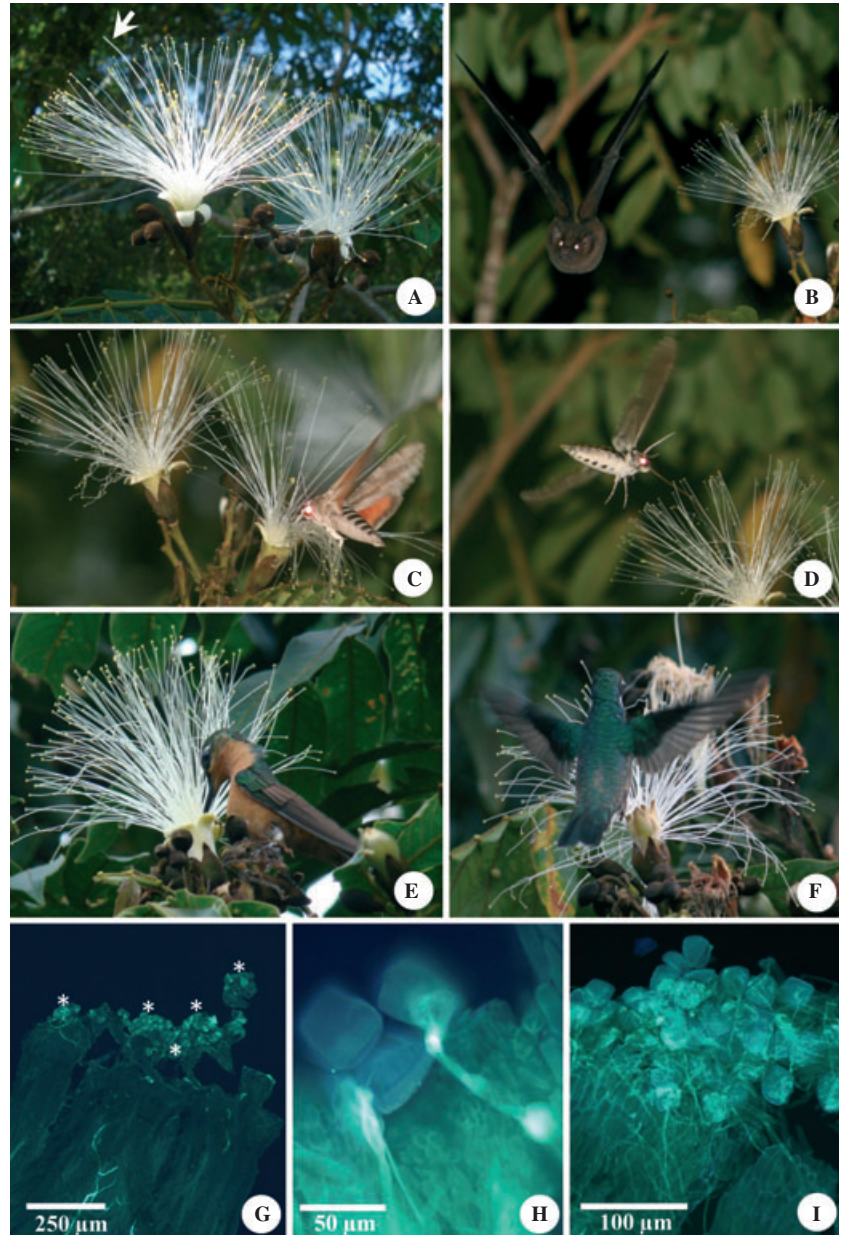


Fig. 2. *Inga sessilis* flower morphology, visitors and pollen germination in a montane ombrophilous dense forest at Núcleo Santa Virginia, Serra do Mar State Park–SP. A: Inflorescence and flower morphology. Note the prominent style (arrow) among the stamens. B: *Glossophaga soricina* after visiting a flower. C: Short-tongued *Erinnyis ello* resting on the stamens while accessing nectar. D: Long-tongued hawkmoth *Manduca brasiliensis* hovering during a visit. E: A perched female *Clytolaema rubricauda* while taking nectar. F: A female *Clytolaema rubricauda* approaching a flower. G: Polyads germinating (asterisks) on the stigma of flowers pollinated under natural conditions. H: Pollen tubes of a self-pollinated flower. I: Pollen tubes of a cross-pollinated flower.

opened flowers nectar was sucrose-dominant, becoming hexose-dominant 4–5 h after flower opening, and hexose-rich during the following hours (Table 2).

Pollinators and compatibility system

Inga sessilis flowers were visited throughout their life span, with bats and hawkmoths as the main nocturnal visitors (Fig. 2B–D), and hummingbirds as the main diurnal visitors (Fig. 2E and F). There was a large variation in visitation frequencies among nights, as well as among years for both groups of visitors (Fig. 4). In February 2009 a total of 140 visits by hawkmoths and 71 by bats was recorded after 2 h of observation (22:00 to 00:00 h). When these visits were analysed through observation periods of 15 min., 6–37 hawkmoth visits and 2–18 bat visits were recorded in each period

(Fig. 4). However, during February 2011 we recorded only zero to three hawkmoths and zero to two bat visits every 15 min of observation (Fig. 4). Hawkmoth visits began early in the evening, just after the sunset, while the first bat visit was recorded after 20:00 h, but visitation frequencies of both groups increased later (after 20:00 h). *Glossophaga soricina* (Pallas) was the most common bat species visiting *I. sessilis* (Fig. 2B), and at least two unidentified bat species visited the flowers occasionally. Bats invariably contacted anthers and stigma when visiting *I. sessilis* flowers, and so did short- and long-tongued hawkmoths during their visits (Fig. 2C and D). Based on direct observations of pollinators and pollen load analysis of light-trapped hawkmoths, 15 species were recorded carrying *I. sessilis* polyads on their bodies, wings and tongues (Table 3). Moth proboscis length of these species ranged between 2.6 and 7.1 cm, all shorter than the operative

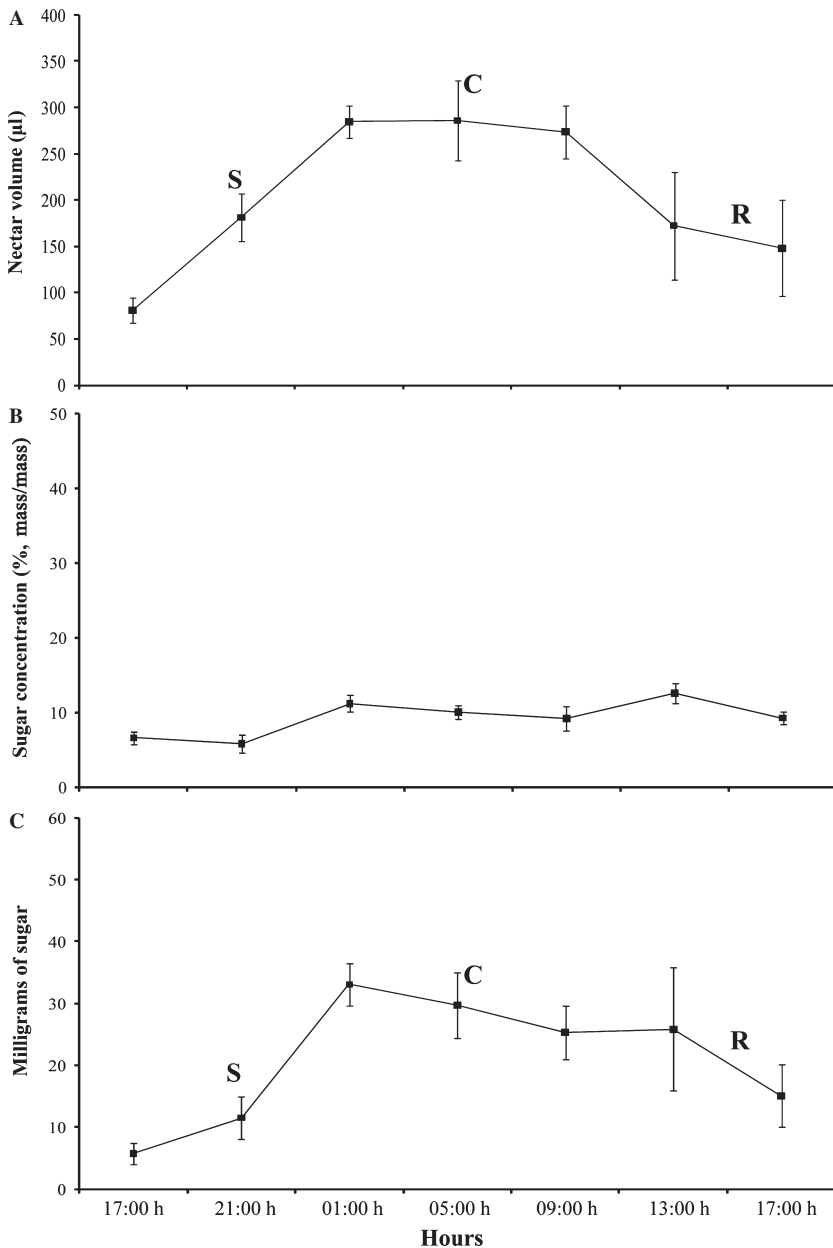


Fig. 3. Nectar features of *Inga sessilis* over the flower life span in a montane ombrophilous dense forest at the Núcleo Santa Virginia, Serra do Mar State Park–SP. A: Nectar volume; (B) nectar sugar concentration; and (C) milligram of nectar sugars. Symbols represent mean \pm SE. S = Secretion period; C = Cessation period; R = Resorption period.

flower length (Table 3), and their abundance partially coincided with *I. sessilis* flowering phenology, mainly during the two flowering peaks (Fig. 1).

Hummingbirds were the main diurnal visitors (Fig. 2E and F). Four species were observed visiting *I. sessilis* flowers: *Clytolaema rubricauda* (Boddaert), *Florisuga fusca* (Vieillot), *Leucochloris albicollis* (Vieillot) and *Thalurania glaucopis* (Gmelin). Between eight and 67 hummingbird visits were recorded during every 15 min of observation (Fig. 4), and visits began before flowers were fully open, anthers were dehiscent and stigma receptive. As nectar was available during the flower opening, they forced their bills among stamens to reach the nectar. A single hummingbird could visit two to 15 flowers of the same plant in sequence, often not contacting anthers and stigma, which occurred either when they landed or hovered beside the flower to take nectar (Fig. 2E).

However, during hovering visits over the flowers (Fig. 2F), anthers and stigma were usually contacted. *Bombus* species were commonly observed visiting *I. sessilis* flowers, but due to their small size and behaviour on flowers they did not contact anthers and stigma. Some diurnal and less frequent hummingbird-moths, *Aellopos* spp., also visited *I. sessilis* flowers, acting as occasional pollinators.

More than 80% of the flowers exposed to pollinators in natural conditions were pollinated, with one to five polyads adhered on the stigmatic surface (Fig. 2G). However, <5% of flowers set fruit (Table 4), despite the massive blooming. Experiments of controlled pollination indicated that *I. sessilis* is self-incompatible and has an ISI of 0.05, although pollen tube germination was observed in both self- and cross-pollination treatments (Fig. 3H and I). The incompatibility reaction probably occurs at ovary level, as pollen tubes reached

Table 1. Nectar removal effects on total nectar production of *Inga sessilis* flowers in a montane ombrophilous dense forest at Núcleo Santa Virginia, Serra do Mar State Park–SP. Data represent mean \pm SD of volume (μ l), concentration (% mass/mass) and (mg of nectar sugar produced per flower (n = 5 flowers per set of flowers sampled at each time).

set of flowers	sampling time (h)							total amount produced*
	17:00	21:00	01:00	05:00	09:00	13:00	17:00	
7								
mg	5.76 \pm 3.74	7.61 \pm 4.11	9.64 \pm 5.02	8.54 \pm 3.77	7.80 \pm 2.17	3.83 \pm 5.10	1.37 \pm 1.87	44.91 \pm 10.50
μ l	81.20 \pm 30.44	135.80 \pm 45.83	143.00 \pm 38.08	123.80 \pm 21.55	106.4 \pm 47.5	38.4 \pm 51.43	14.80 \pm 20.38	643.4 \pm 118.11
%	6.60 \pm 1.82	5.40 \pm 1.52	6.40 \pm 2.07	6.60 \pm 2.70	8.17 \pm 3.13	6.68 \pm 6.61	3.60 \pm 4.98	a
6								
mg		11.53 \pm 7.70	9.59 \pm 9.01	8.75 \pm 8.49	6.81 \pm 3.82	0.19 \pm 0.27	1.20 \pm 2.68	37.97 \pm 26.98
μ l		181.00 \pm 57.20	121.20 \pm 64.74	108.20 \pm 53.36	93.20 \pm 38.98	13.00 \pm 24.68	0.60 \pm 1.34	518.0 \pm 174.11
%		5.80 \pm 2.59	6.20 \pm 3.70	6.60 \pm 3.78	6.80 \pm 3.35	6.40 \pm 6.19	0.04 \pm 0.08	a
5								
mg			33.08 \pm 7.53	10.64 \pm 3.50	8.89 \pm 2.77	3.31 \pm 1.77	0.61 \pm 0.59	56.53 \pm 7.28
μ l			284.60 \pm 38.51	103.80 \pm 22.50	88.20 \pm 25.79	29.00 \pm 15.73	10.00 \pm 8.86	515.60 \pm 50.15
%			11.20 \pm 2.49	9.80 \pm 1.79	9.80 \pm 1.64	11.00 \pm 0.71	5.00 \pm 3.16	b
4								
mg				29.72 \pm 10.48	13.07 \pm 1.81	3.29 \pm 1.70	0.49 \pm 0.54	50.63 \pm 10.43
μ l				286.00 \pm 85.91	129.5 \pm 22.94	30.50 \pm 17.37	9.25 \pm 7.27	455.25 \pm 83.40
%				10.00 \pm 1.83	10.00 \pm 2.45	10.75 \pm 1.26	6.00 \pm 2.71	b
3								
mg					25.33 \pm 9.59	2.34 \pm 1.15	0.32 \pm 0.37	27.99 \pm 8.94
μ l					273.60 \pm 63.78	24.20 \pm 13.08	6.00 \pm 7.97	303.80 \pm 61.51
%					9.20 \pm 3.63	9.60 \pm 2.61	3.60 \pm 3.58	a
2								
mg						25.82 \pm 22.24	0.46 \pm 0.50	26.27 \pm 22.99
μ l						172.40 \pm 129.99	5.60 \pm 5.32	178.0 \pm 129.67
%						12.60 \pm 2.97	6.20 \pm 3.90	a
§ 1								
mg							15.06 \pm 12.17	15.06 \pm 10.08
μ l							148.00 \pm 126.61	148.00 \pm 126.61
%							9.25 \pm 1.53	

*Different letters in lines represent significant differences among sets by Tukey *post-hoc* test, $P < 0.05$.

§ The last group was not considered in the statistical analyses because it is included within the absorption period (see Material and Methods for more details).

Table 2. Nectar sugar composition of *Inga sessilis* flowers in a montane ombrophilous dense forest at Núcleo Santa Virginia, Serra do Mar State Park – SP, at different times of anthesis. Classification of nectar type followed Baker & Baker (1983), in which: SD = sucrose-dominant nectar; HD = hexose-dominant nectar; HR = hexose-rich nectar.

time of removal	amplitude		sugar composition			proportion		type
	volume (μ l)	concentration (%)	sucrose (S)	fructose (F)	glucose (G)	S/(F+G)		
17:00–18:00	22–58	17.5–21.5	36.03	36.77	27.21	0.563	SD	
21:00–22:00	18–37	13.5–18.5	9.29	53.58	37.14	0.102	HD	
07:00–08:00	50–194	10.5–17.0	5.17	58.64	36.18	0.055	HR	

the ovary even in self-pollinated pistils. Fruit set in self-pollinated pistils was close to zero and no fruits were formed by apomixis (Table 4).

Efficiency of nocturnal versus diurnal pollinators

Analysing the results of both 2010 and 2011 together, pollinator exclusion experiments showed differences in the relative contribution of diurnal and nocturnal visitors to the fruit set

[diurnal: 1.2% (four fruits) and nocturnal: 3.6% (12 fruits), $\chi^2 = 4.0$, $df = 1$, $P = 0.045$]. Considering each year separately, the relative contribution of nocturnal pollinators to fruit set was also higher. In 2010 the percentage of flowers that set fruits due to activity of diurnal pollinators was 2.3% (two fruits), and nocturnal pollinator activity resulted in 4.5% fruit set (four fruits). In 2011 the percentages for diurnal and nocturnal pollinator activity were, respectively, 0.81% (two fruits) and 3.25% (eight fruits).

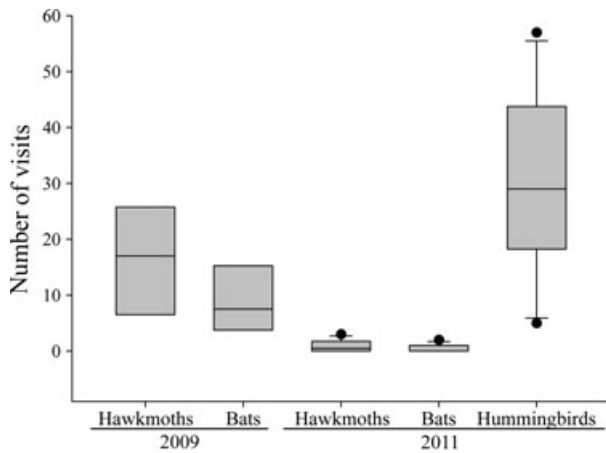


Fig. 4. Number of hawkmoth, bat and hummingbird visits to *Inga sessilis* flowers in a montane ombrophilous dense forest at the Núcleo Santa Virginia, Serra do Mar State Park–SP. Observations were made during February 2010 and 2011 between 16:00 and 01:00 h, and number of visits is plotted at 15-min intervals.

DISCUSSION

Phenology and floral biology

The higher flowering intensity of *I. sessilis* during the wettest period follows the general pattern of Atlantic rain forest trees (Morellato *et al.* 2000). This pattern was also recorded in non-tree bat-pollinated plants in another highland Atlantic rain forest area in southeast Brazil (Sazima *et al.* 1999). Additionally, the abundance of hawkmoth species effectively carrying *I. sessilis* polyads partially coincided with *I. sessilis* flowering intensity, especially during periods of higher blooming (Fig. 1). However, hawkmoth activity was still high even during the months of lowest *I. sessilis* flowering intensity. This pattern of synchrony (at least in certain periods of

the year) between hawkmoth abundance and blooming of *Inga* species was recently recorded in an Atlantic rain forest in northeast Brazil (Cruz-Neto *et al.* 2011). Nonetheless, hawkmoth abundance is highly associated with other factors, such as temperature, humidity and rainfall (Amorim *et al.* 2009). On the other hand, typical hummingbird-pollinated floras in highland Atlantic rain forest areas in southeast Brazil (Buzato *et al.* 2000; Freitas *et al.* 2006) have many species with continual flowering pattern such as *I. sessilis*.

Flower scent probably plays a small role in *I. sessilis* pollinator attraction (Koptur 1983). The showy androecium acts as the main flower display in the attraction of visitors, especially during the blooming peaks when *I. sessilis* can be characterised as a typical mass-flowering species (*sensu* Gentry 1974). The PP/O ratio found in *I. sessilis* indicated that a single polyad is potentially able to fertilise all ovules of a flower, and the remarkable P/O ratio is two to nine times higher than those in other *Inga* species (Koptur 1984). Such a P/O ratio is common to self-incompatible species, especially in mass-flowering plants, which are prone to high rates of pollen limitation due to excessive geitonogamy (Cruden 1977; Koptur 1984).

Nectar: secretion pattern, removal effects and chemical composition

The period of highest nectar secretion in *I. sessilis* was related to the highest pollinator frequencies on flowers. The dynamics of nectar production seem to be directly related to pollinator activity (Zimmerman & Pyke 1986; Torres & Galetto 1998; SanMartín-Gajardo & Sazima 2005). Recent studies have demonstrated that the period of nectar secretion can play an important role in hawkmoth visitation activity on flowers (Guerenstein *et al.* 2004a; Thom *et al.* 2004), since flowers produce high levels of CO₂ during nectar secretion, which can be used by hawkmoths as a clue to nectar availability (Guerenstein *et al.* 2004a,b; Thom *et al.* 2004; Goyret *et al.* 2008).

Table 3. Hawkmoth species recorded either visiting flowers or carrying *Inga sessilis* polyads in a montane ombrophilous dense forest at the Núcleo Santa Virginia, Serra do Mar State Park–SP. Visitation data are based on the pollen load of light-trapped moths throughout a period of 1 year.

species	month												proboscis
	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec	mean ± sd
<i>Adhemarius eurythenes</i> (Felder, 1874)	1	9	3	–	7	7	27	14	11	–	4	–	2.60 ± 0.15
<i>Adhemarius gannascus</i> (Stoll, 1790)	–	–	–	2	–	–	2	1	3	1	4	–	2.65 ± 0.15
<i>Erinnyis ello</i> (Linnaeus, 1758)*	2	2	–	–	–	–	1	–	–	–	2	–	3.61 ± 0.19
<i>Erinnyis oenotrus</i> (Cramer, 1780)	–	4	–	–	–	–	–	–	–	–	2	4	3.98 ± 0.35
<i>Eumorpha analis</i> (Rot. & Jor, 1903)*	2	3	–	–	–	–	–	–	–	–	–	–	4.92 ± 0.80
<i>Hemeroplanes longistriga</i> (Rot. & Jor, 1903)	–	–	–	–	1	1	2	2	1	–	–	–	3.97 ± 0.18
<i>Manduca diffissa</i> (Butler, 1871)*	–	6	4	–	–	–	–	–	–	3	11	1	7.14 ± 0.35
<i>Manduca florestan</i> (Stoll, 1782)*	–	6	3	–	–	–	–	–	–	–	3	1	6.13 ± 0.49
<i>Manduca brasiliensis</i> (Jordan, 1911)*	–	1	9	–	–	–	–	–	1	2	1	–	7.11 ± 0.75
<i>Pachylia ficus</i> (Linnaeus, 1758)	3	1	–	–	–	–	–	2	3	1	–	1	4.83 ± 0.33
<i>Pachylioides resumens</i> (Walker, 1856)*	3	2	2	1	–	–	1	–	1	1	1	–	2.67 ± 0.23
<i>Xylophanes ceratomioides</i> (Gr. & Rob, 1866)	6	2	1	–	–	–	–	–	3	4	4	2	3.60 ± 0.21
<i>Xylophanes chiron</i> (Drury, 1773)	1	1	–	1	–	1	–	–	–	–	3	–	4.71 ± 0.39
<i>Xylophanes isaon</i> (Boisduval, 1875)	–	14	2	–	–	–	3	2	7	–	9	–	2.61 ± 0.35
<i>Xylophanes thyelia</i> (Linnaeus, 1758)	–	2	1	4	14	3	2	2	4	1	8	1	3.78 ± 0.45

*Species observed both visiting *I. sessilis* flowers and carrying polyads.

Table 4. Percentage of fruit set of *Inga sessilis* after pollination treatments in a montane ombrophilous dense forest at the Núcleo Santa Virginia, Serra do Mar State Park – SP.

pollination treatments	flowers	fruits (%)
cross-pollination	80	21 (26.3)
hand-self-pollination	80	1 (1.25)
autonomous self-pollination	100	0 (0)
emasculatation (agamospermy)	100	0 (0)
control (natural conditions)	1054	48 (4.55)

The results of nectar removal experiments suggest that pollinator activity on flowers may increase the total amount of nectar secreted. Similar patterns, suggesting an increase in the total amount of nectar production due to pollinator activity, have also been recorded for other *Inga* species (Koptur 1994). It is interesting that in *I. sessilis*, nectar production increases to higher rates when it is removed by nocturnal flower visitors, more than when it is removed by diurnal ones (Table 1), suggesting a closer relationship with nocturnal pollinators.

Nectar resorption was characterised by a constant decrease in nectar volume and amount of sugars. As sugar production in *I. sessilis* can reach up to 56 mg per flower and one single plant can produce hundreds of flowers per day over many weeks, nectar resorption may serve as an important strategy to save and reallocate resources to the production of new flowers, fruits and seeds (Pyke 1991; Nepi & Stpiczynska 2008; Agostini *et al.* 2011).

During the night, the nectar sugar composition of *I. sessilis* seems better to fit bat requirements (Faegri & van der Pijl 1980; Von Helversen 1993; Koptur 1994), as the nectar composition of typical hummingbird- and hawkmoth-pollinated flowers are dominated by sucrose (Baker & Baker 1983; Koptur 1994). However, *I. sessilis* also presents sucrose-rich nectar during the first hours of the flower's life span (before sunset), a time when hummingbirds were the most frequent visitors, and after sunset when hawkmoths are active. Nevertheless, hawkmoths remain active during most of the night when nectar becomes hexose-dominant/rich. In this sense such nectar sugar features encompass the preferences of diurnal and nocturnal pollinators.

Pollinators and compatibility system

As recorded for other *Inga* species (Koptur 1984; Cruz-Neto *et al.* 2007), our results indicate that *I. sessilis* is self-incompatible and pollinator dependent for sexual reproduction. Previous studies on *Inga* species have shown their dependency on long-distance pollinator movements among plants for fruit set (Koptur 1984). In such species, cross-pollination among plants within 0.5 km leads to fewer fruits than crosses between plants more than 1.0 km apart (Koptur 1984). Despite flowering continuously throughout the year and blooming massively during the flowering peaks, where one single plant can produce up to 300 flowers per day, fruit set in *I. sessilis* in natural conditions was as low as 5%. On the other hand, our analyses revealed that more than 80% of flowers were effectively pollinated, presenting polyads germinating on the stigmatic surface. This indicates that *I. sessilis*

visitors very likely promote self-pollination in more than 75% of flowers. Similar patterns of low fruit set have been observed in at least 12 species of *Inga*, despite the relatively high frequency of visitors (Koptur 1984; Cruz-Neto *et al.* 2007, 2011).

Such a low fruit set was also observed in *Caryocar brasiliensis* Camb. (Caryocaraceae), a common tree of the Cerrado vegetation in Central Brazil, which has similar flowers in terms of morphology as *I. sessilis* and also share as pollinators, hawkmoths, bats and hummingbirds (Gribel & Hay 1993). Recent genetic analyses have shown that the fruit set in *C. brasiliensis* mostly results from self-pollination and short-distance pollen dispersal (up to 200 m) among individuals (Collevatti *et al.* 2010). Assuming similar trends in *I. sessilis*, the low fruit set may be a result of pollen limitation, not because of absence of pollinators, but due to low quantities of cross-pollen. In this sense, pollinator behaviour, in conjunction with self-incompatibility, are probably the main causes of the low fruit set recorded in *I. sessilis*.

Efficiency of diurnal versus nocturnal pollinators

Hummingbirds are common pollinators of many *Inga* species (Koptur 1984; Pennington 1997). Based on our qualitative observations, the frequency of visits of these birds in *I. sessilis* throughout the year is higher and seems to be much more constant and reliable than the visits of nocturnal pollinators. However, hummingbird contribution to the reproductive success of *I. sessilis* is significantly smaller than that of nocturnal pollinators. All hummingbird species observed visiting *I. sessilis* belong to the Trochilinae sub-family, which are characteristically territorial, especially when resources are aggregated (Feinsinger 1976; Justino *et al.* 2012), as is the case of mass-flowering *Inga* species. In addition to their territorial behaviour, hummingbirds often perform illegitimate visits on *I. sessilis* flowers, a foraging behaviour that greatly limits pollen dispersal over long distances and also between plants, both of which are required to optimise fruit production in *Inga* species (Koptur 1984).

Bats and hawkmoths are the main pollinators of *I. sessilis*, contributing significantly more to fruit production than diurnal pollinators. Based solely on classical concepts of the pollination syndrome, *I. sessilis* could be considered only as chiropterophilous (Vogel 1969; Faegri & van der Pijl 1980). Although it was not possible to separately quantify the relative contribution of bats and hawkmoths to the reproductive success of *I. sessilis*, some features of the flowers also indicated sphingophily (Vogel 1969; Faegri & van der Pijl 1980). But both groups of animals perform legitimate visits to flowers and both transport pollen among plants. Glossophaginae bats, however, may present territorial behaviour, which favours geitonogamy and short-distance pollen flow (Gribel & Hay 1993). In this sense, assuming such a foraging behaviour by bats in a self-incompatible species such as *I. sessilis*, pollen transport could be limited, thus impairing fruit production.

On the other hand, despite the large number of hawkmoth species visiting and effectively carrying *I. sessilis* polyads, in general these insects visit many flowers in a single plant, limiting pollen flow among individuals. However, our analyses of the pollen load of the hawkmoth fauna collected at NSV

(F.W. Amorim, A.M.S. Corrêa, M.A.V. Cruz-Barros, M. Sazima, unpublished observations) indicated that some moth species, including *I. sessilis* visitors, can move over long distances in the Atlantic rain forest. This analysis revealed that some hawkmoth species were carrying *Tocoyena bullata* Mart. (Rubiaceae) pollen, a species exclusive to coastal Restinga vegetation in the lowland forest, at least 15 km from the collection site. Hence, some hawkmoth species can transport pollen over long distances, which is more effective for fruit production of *Inga* species (Koptur 1984).

The question then is, if bats and hawkmoths are the most effective pollinators, why does *I. sessilis* produce nectar during the day, providing such an expensive floral reward to less effective pollinators? Based on the pollen tube growth analysis of stigmas from flowers exposed to both diurnal and nocturnal pollinators, we observed that they deposit polyads in more than 80% of *I. sessilis* flowers. Nonetheless, fruit production under natural conditions is about 5%, approximately the same percentage as the contribution of both diurnal and nocturnal pollinators together to the fruit set. Moreover, when fruit production is pollen-limited (as in this case, limited by pollen quality) and the reliability of primary pollinators varies annually, selection favours more generalised pollination (Waser *et al.* 1996; Fleming *et al.* 2001). Thus hummingbirds act as important pollinators of *I. sessilis* as they seem to be more constant visitors, whereas bats and hawkmoths may be inconsistent. In this sense, *I. sessilis* has a clear mixed and complementary pollination system, in which some pollinators are always available (Miyake & Yahara 1998, 1999; Wolff *et al.* 2003; Giménez-Benavides *et al.* 2007; Maruyama *et al.* 2010).

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