



Obligate association of an oligolectic bee and a seasonal aquatic herb in semi-arid north-eastern Brazil

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Hydrocleys martii (Limnocharitaceae) is an annual aquatic herb common in ephemeral isolated ponds in semi-arid north-eastern Brazil. We studied pollination of *H. martii*, emphasizing reproductive success and association with oligolectic pollinators. The yellow flowers bear a central cone of staminodes that encloses the fertile stamens and four free carpels. The self-incompatible species depends on pollinators to set fruits. In 25 temporary water bodies in five Brazilian states, *Protodiscelis palpalis* bees (Colletidae, Paracolletinae) were the unique effective pollinators of *H. martii* and, in 18 of these, the sole flower visitors. Females of this narrowly oligolectic species show adapted behaviour to access the pollen chamber in the flower centre. Females removed more than 80% of the 480 000 pollen grains in only 2 h from the flowers but maintained a high visitation frequency almost until flower senescence. In this highly specialized plant-pollinator system, on average, 1.6% of the pollen grains reached the stigmatic surface and 9.6% remained uncollectible in the flowers. In the absence of *P. palpalis*, flowers set almost no seeds, indicating reproductive dependence on the oligolectic species. This pioneer pollination study of a species of Limnocharitaceae provides evidence of a close relationship of the family to species of *Protodiscelis*, their specific pollinators. © 2011 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2011, **102**, 355–368.

ADDITIONAL KEYWORDS: Alismatidae – Limnocharitaceae – Colletidae – *Hydrocleys* – mutual reproductive dependence – pollination – *Protodiscelis*.

INTRODUCTION

Seasonal aquatic plants confined to lentic water bodies generally exhibit sharply delimited distribution patterns within the *terra firme* matrix. The majority of such plant species have a high capacity for long distance seed dispersal and thus often present a broad geographic distribution (Barrett, Eckert & Husband, 1993; Figuerola & Green, 2002). Several aquatic species are either primarily or secondarily dispersed by water birds and their seeds can be carried by currents and even fish (Gottsberger, 1978; Barrett *et al.*, 1993; Charalambidou & Santamaria,

2002; Figuerola & Green, 2002; Pollux *et al.*, 2006). The fruits, in general, bear numerous tiny seeds to optimize the infrequent dispersal events (Barett & Husband, 1991; Barrett *et al.*, 1993). This type of distribution and adaptations in such environments often imply a generalist pollination system (Johnson & Steiner, 2000), and autogamous or vegetative reproduction is common (Baker, 1974; Waser *et al.*, 1996; Jacquemyn *et al.*, 2006; Waser & Ollerton, 2006; Pollux *et al.*, 2007).

The Caatinga of north-eastern Brazil, a tropical dry forest, is characterized by succulents and spiny xerophytic woody plant species, shedding their leaves during the severe annual drought (Andrade-Lima, 1961; Sampaio, 1995; Prado, 2003). After the few heavy and irregular rainfalls of the wet season of the Caatinga region, depressions in the landscape fill

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with water and form shallow ponds, and seasonal rivers carry water for a short time (Maltchik & Medeiros, 2006). The ponds may last for a few weeks up to a few months, depending on their depth and the amount of rainfall, and are temporarily colonized by aquatic plants (Maltchik & Pedro, 2001), mainly comprising annual species (Barrett & Husband, 1997).

Hydrocleys martii Seub. (Limnocharitaceae) is one of the aquatic herbs that is commonly found in the ephemeral ponds of the Caatinga (Haynes & Holm-Nielsen, 1992). However, populations of *H. martii* are always isolated, sometimes being separated kilometres from each other by the Caatinga matrix, common to aquatic plants in the Caatinga (Barrett, 1985). There is no information referring to flower biology or pollination of Limnocharitaceae. The yellow disk shaped flowers suggest pollination by bees. Data originating from bee-plant surveys, nevertheless, showed that bees of the species *Protodiscelis palpalis* (Ducke, 1908) (Colletidae, Paracolletinae) were extraordinarily frequent flower visitors of *H. martii* (C. Schlindwein *et al.*, unpubl. data). All New World species of Paracolletinae studied until now are oligolectic (Schlindwein, 2004), meaning that females collect pollen in host plants of the same genus or family (Robertson, 1925; Cane & Sipes, 2006). The present study aimed to investigate the breeding system of *Hydrocleys martii* and the characteristics of its floral biology; the characteristics of the association of *H. martii* with flower visitors; and whether there is reproductive dependence of *H. martii* and *P. palpalis*.

MATERIAL AND METHODS

STUDIED SPECIES

Hydrocleys is one of the three genera of Limnocharitaceae Takhtajan 1954, a small family of aquatic or semi-aquatic plants with eight recognized species, associated with basal monocotyledons (Cronquist, 1981; Haynes & Holm-Nielsen, 1987, 1992; Koehler & Bove, 2004). According to Haynes & Holm-Nielsen (1989, 1992), the family probably originated in north-east Brazil and has its distribution centre in the states of Pernambuco and Paraíba. *Hydrocleys martii* is distributed from Uruguay to the Brazilian states of Mato Grosso, and Pará, being especially abundant in north-eastern Brazil. The roots are fixed in the substrate and the leaves float on the water surface (Haynes & Holm-Nielsen, 1992). The inflorescence, a cymose umbel, bears 30 ± 4 mm wide yellow flowers with a dark centre. The flowers emit a relatively strong cockroach-like odour. The androecium is composed of staminodes that surround the fertile stamens, whereas the gynoecium comprises four free

carpels containing numerous ovules and a short apical stigma (Haynes & Holm-Nielsen, 1992). This aquatic species is restricted to permanent or seasonal lentic water bodies such as shallow ponds and only rarely grows on river margins with weak currents. In regions with marked seasonal rainfalls, such as the Caatinga, where the growth period is restricted to a few months per year, flowering depends on the local rainfall regime and occurrence of the species is insular.

STUDY AREA

The study was conducted from June 2005 to December 2007 at the Nature Reserve 'Parque Ecológico João Vasconcelos Sobrinho' (Brejo dos Cavalos, 08°22'09"S; 36°05'00"W) municipality of Caruaru, Pernambuco, Brazil. The park covers 359 ha with altitudes varying in the range 820–950 m. Mean annual rainfall oscillates within the range 650–900 mm, and mean annual temperature is 24 °C (CPRH, 1994). The area is occupied by a rather preserved and diverse tropical montane rainforest (in north-eastern Brazil called 'Brejo de Altitude') containing trees, lianas, epiphytes, and ferns (Andrade-Lima, 1961). In semi-arid north-eastern Brazil, this forest type has discontinuous distribution, occurring exclusively on mountain tops within the Caatinga region. Individuals of *H. martii* were common in the drainage canal of an irrigated plantation of chayote, *Sechium edule* (Jacq.) Swartz. (Cucurbitaceae) within the Nature Reserve.

We made several additional observations and collections of flower visitors of *H. martii* in locations within the drier Caatinga, which is characterized by a more severe drought and lower annual precipitation (< 600 mm) than that of the Caruaru site, in the states of Pernambuco, Rio Grande do Norte, Piauí, Sergipe, and Ceará, which summed to a total of 25 temporary water bodies (Fig. 1). The sites with their respective predominant vegetation were: São Lourenço da Mata (Tapacurá), Pernambuco (7°58'12"S; 35°6'53"W), semideciduous forest, one pond; Sanharó, Pernambuco (8°21'39"S; 36°33'33"W) Caatinga, two ponds; Pedra, Pernambuco (8°36'45"S; 36°51'11"W) dense Caatinga scrub, three ponds 2 km distant from each other; Buíque, Pernambuco (8°37'46"S; 37°10'6"W), Caatinga with evergreen shrubs, one pond; Cabrobó, Pernambuco (8°29'36"S; 39°17'37"W) dense Caatinga scrub, one pond; Serra Negra do Norte, Rio Grande do Norte (6°39'47"S; 37°24'1"W) arboreous Caatinga, 12 ponds, minimum distance between water bodies 1 km; Caracol, Piauí (9°17'17"S; 43°19'44"W) transition of Caatinga to Cerrado, one pond; Caucaia, Ceará (3°03'03"S; 35°03'02"W) open arboreous Caatinga, one pond;

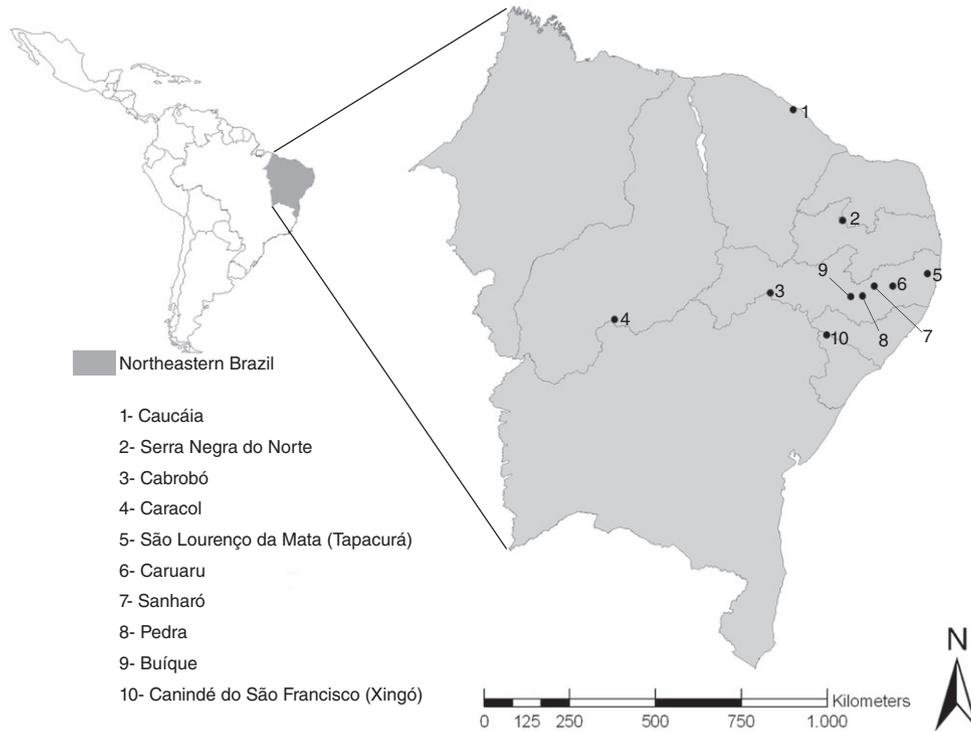


Figure 1. Locations of samplings of flower visitors of *Hydrocleys martii* in North-eastern Brazil.

Canindé do São Francisco (Xingó), Sergipe (9°38'31"S; 37°47'18"W) open arboreous Caatinga, one pond.

FLOWER BIOLOGY AND MORPHOLOGY

Flower biology was studied in the Caruaru population. We chose 15 *H. martii* individuals; for each, one flower was dissected and was measured with respect to its diameter, length of the petals and sepals, and the height of the staminode cone, using a digital calliper. The number of staminodes and fertile stamens were counted. Flowers and flower buds were photographed with a digital camera equipped with a 100 mm macro lens and on a stereomicroscope (Stemi SV8; Zeiss). Moreover, the fruits, fruit dehiscence, and external morphology of the seeds were examined.

Anthesis was accompanied in 100 previously bagged flowers and determined floral longevity, the time of nectar and pollen availability, circadian rhythm, and nastic movements of the stamens. Moreover, we marked other 50 flowers, accessible to flower visitors. The time of stigmatic receptivity was verified in the field with hydrogen peroxide and potassium permanganate (Kearns & Inouye, 1993). In ten flowers, we determined the number of pollen grains per flower, using a Neubauer chamber (haemocytometer, appropriate for counting particles smaller than 100 µm). The anthers were smashed inside essay tubes, to which 1 mL of glycerinated lactic acid 3 : 1 was added. After homo-

genization in a vortex stirrer, an aliquot was counted in the Neubauer chamber. Pollen from ten fresh flowers (1 h after opening) was added to a solution of Acetic Carmin 5% to determine pollen viability. The equatorial diameter was measured in 20 pollen grains from four preparations. The ovaries of ten flowers from different plants were dissected and the number of ovules was counted under a stereomicroscope.

The speed of the removal of pollen grains by the flower visitors was determined in 60 individually marked flowers in the field. At each hour, ten flowers were allotted, collected and fixed in formaldehyde, glacial acetic acid and alcohol (70%, 5 : 5 : 90, v/v). The number of pollen grains remaining in the flower was counted for each hour of anthesis. The flowers were dissected in laboratory and anthers were inserted in centrifugation tubes. The remainder of the flower parts, petals, sepals, and staminodes was washed with neutral concentrated detergent and deionized distilled water. Each tube was sonicated in a vortex stirrer for 10 min. After removal of the floral parts from the centrifuge tube, the detergent was centrifuged for 45 min at 3500 r.p.m (1852.2 g.). The supernatant was removed and pollen grains were diluted in 1 mL of a 3 : 1 solution of glycerine and lactic acid. The solution was set again in the vortex stirrer for 5 min, and an aliquot transferred to the Neubauer chamber, under which the grains were counted. The mean number of the pollen grains left in the flowers were

compared by a one-way analysis of variance (ANOVA) after verification of the assumptions of the tests. STATISTICA, version 6.0 (Statsoft Inc.) was used for these analyses. At the end of the anthesis, we collected the stigmas from five flowers, included them in fuch-sine stained glycerine, and counted the pollen grains attached to them.

GROWTH AND VEGETATIVE REPRODUCTION

Thirty *H. martii* individuals were transferred to an experimental garden (meliponary of Rodrigo O. C. Carvalho in the municipality of Camaragibe). The area is inserted within the Atlantic Rainforest domain in the state of Pernambuco, where *P. palpalis* does not occur. The experimental area was surrounded by seminatural forest vegetation. The plants were cultivated in water tanks and 22 young inflorescences were monitored daily over 30 days. We accompanied the orientation of the flowers from bud to fruit development and determined the growth pattern. From these daily observations, the vegetative propagation was analyzed.

BREEDING SYSTEM

The breeding system was determined in the Caruaru population through a controlled pollination experiment with previously bagged flowers of different individuals: 35 flowers were hand cross-pollinated, 35 were hand self-pollinated and 75 were maintained bagged (spontaneous self-pollination without manipulation). From these, 30 hand cross-pollinated, 34 and self-pollinated and 74 non-manipulated bagged flowers were recovered. Another 55 flowers, accessible to pollinators were marked as open-pollinated controls. Fruit and seed set from all treatments were determined.

The average number of seeds formed after manual cross-pollination was compared with that produced by flowers accessible to flower visitors. After checking the normality of the data, the average number of the seeds was compared by the *t*-test.

FLOWER VISITORS AND DETERMINATION OF EFFECTIVE POLLINATORS

The spectrum of the flower visitors was determined by collecting insects inside flowers with entomological nets from each sampled locality. At the Caruaru and Serra Negra do Norte sites, we determined the frequency of visits from flower opening until senescence. In the remaining localities, two individuals performed intense punctual sampling for 2 h. In Caruaru, two individuals monitored the frequency of flower visitors in seven to nine flowers for 4 days for a total of 34

flowers and 28 h of observation. In Serra Negra do Norte, ten flowers were monitored by one individuals over 7 h of observation. The visits were grouped in 30-min intervals to determine the visitation rate throughout anthesis.

During the observations, we differentiated the species and sex of visitors and recorded whether the bees collected pollen or nectar and whether the visitors came into contact with stigmas and anthers. Effective pollinators were determined by taking into account contacts with stigmas and anthers and the foraging routes of the bees. Moreover, we determined flower fidelity of females from an analysis of scopal pollen loads from five females per species and locality and analyzed the pollen grains attached to the body of ten males, which were chosen randomly among the flower visitors.

Plant vouchers were deposited in Herbarium UFP and bees in the Entomological Collection of the Federal University of Pernambuco.

The duration of female *P. palpalis* flower visits was determined in 200 visits over the anthesis. To test whether the mean duration of a visit was shorter in older, emptier flowers, data were divided into (i) duration of visits from 09.00–11.00 h and (ii) from 12.00–14.00 h. The mean times were compared using a *t*-test and time data were transformed to natural logs (Zar, 1996).

Moreover, we determined the rate of flower damages by *Trigona spinipes* worker bees in 270 flowers for two nonconsecutive days.

REPRODUCTIVE SUCCESS IN THE ABSENCE OF OLIGOLECTIC BEE

To determine fruit set in the absence of the oligolectic bees, 50 plants from the Caruaru population were cultivated at a site in Camaragibe, which is surrounded by secondary Atlantic Rainforest and tropical fruit crops such as *Annona muricata* (Annonaceae), *Malpighia emarginata* (Malpighiaceae), and *Eugenia uniflora* (Myrtaceae), where *P. palpalis* does not occur. In 100 flowers, we determined the spectrum of flower visitors and the frequency of flower visits. Fruit and seed set were compared with that of hand-cross pollinated flowers and flowers accessible to pollinators under the presence of the oligolectic species (Caruaru and Serra Negra do Norte sites).

RESULTS

FLOWER BIOLOGY AND MORPHOLOGY

The flowers of *H. martii* presented an androecium divided into inner fertile stamens and surrounding staminodes inserted at the base of the flower as a long spiral (Fig. 2C, E–H). A flower contained 50–69 sta-

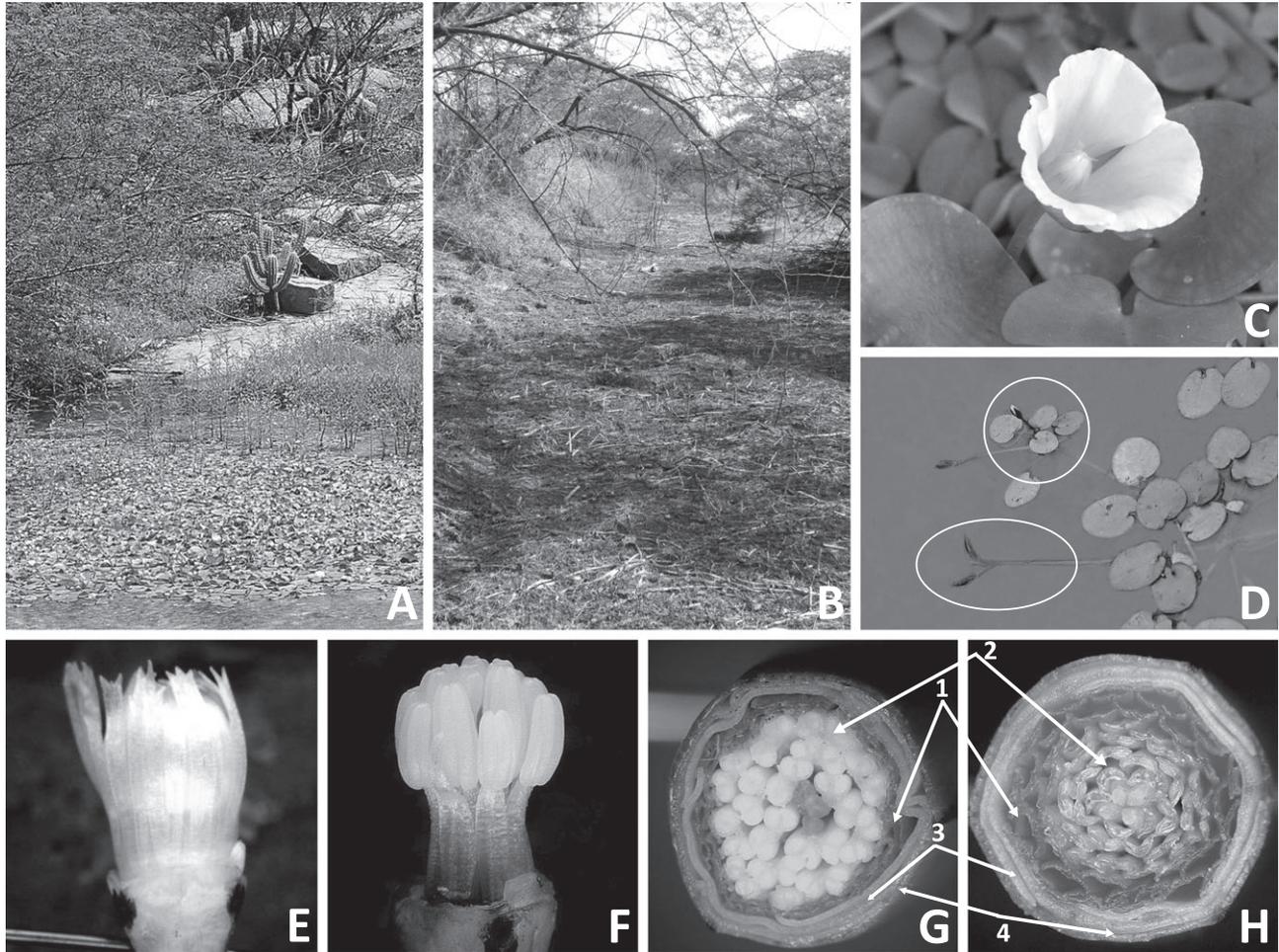


Figure 2. Habitat, flowers and floral buds of *Hydrocleys martii* (Limnocharitaceae). A, pond with *H. martii* in Serra Negra do Norte, Rio Grande do Norte; typical caatinga vegetation in the background. B, pond at the beginning of the dry season. C, flower of *Hydrocleys martii*. D, a plant floating on the water surface; modules with stem, leaves and inflorescence; lower circle: a new module. E, central cone of staminodes, containing fertile stamens and carpels. F, fertile stamens, exposed after removal of the staminodes. G, H, flower buds. G, transverse section at the height of stamens (1, staminodes; 2, stamens; 3, petals; 4, sepals). H, lower section, indicating spiral insertion of staminodes and fertile stamens.

minodes (mean \pm SD: 61.0 ± 6) and 12–24 fertile stamens (19.5 ± 4). The staminodes are longer than the fertile stamens, surround anthers and four free carpels, forming a cone 12.1 ± 1.4 mm long, which contains the pollen grains in a chamber that is not freely accessible from outside (Fig. 2E, F).

The flowers opened simultaneously between 08.45 h and 09.00 h. Bagged flowers closed between 15.30 h and 16.00 h ($N = 100$), and those accessible to flower visitors between 13.30 h and 14.00 h ($N = 50$), two hours earlier than nonvisited flowers. Opening of the flowers took 20–35 min on sunny days and up to 1 h on cloudy days.

The floral buds developed underwater and, on the day before opening, they emerged perpendicularly to the water surface at approximately 15.00 h. The pedicel stretched towards sunlight for 5 h. With the

opening of the flowers on the next morning, the stigmas were already receptive and the anthers dehisced. Soon after flower closure, the pedicels bent downward and, at 06.00 h on the following day, they were already once more submerged ($N = 22$) (Fig. 3).

Hydrocleys martii individuals exhibited a sympodial modular growth pattern. Each module consisted of a lateral born branch containing a terminal inflorescence and four leaves. Each inflorescence carried five to ten flowers (mean 7.5; $N = 22$). Every 3–4 days, a new flower opened and whole inflorescences had longevity of 14–33 days. After the opening of the second flower of an inflorescence, the first pair of leaves developed at the apex of the branch and, after 6–8 days, the second pair of leaves appeared. These modules were repeated continuously until the plants covered the water surface of the ponds. Fifteen to 20

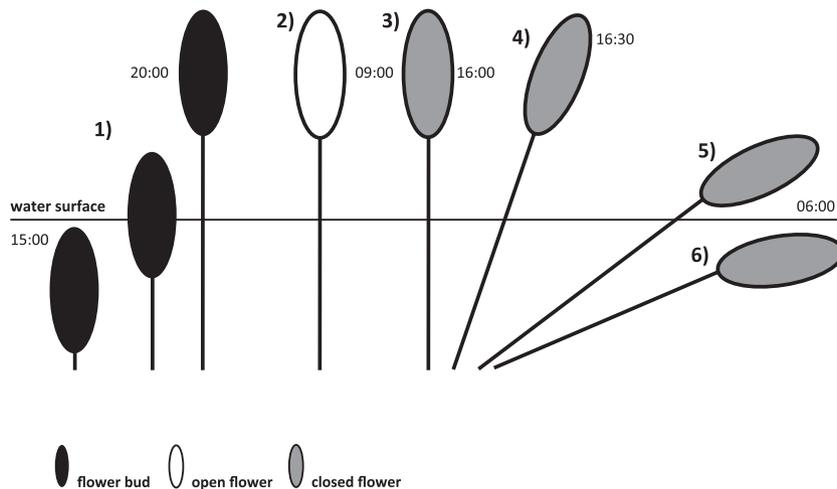


Figure 3. Blooming phases of *Hydrocleys martii*. 1, Vertical prolongation of the pedicel (at around 15.00 h of the day before the opening flower bud emerges from the water surface); 2, the flower opens at 09.00 h and closes around 13.00 h; 3, closed flower; 4, 5, bending down of closed flowers (15.00 h); at around 18.00 h, the flowers reach the water surface; 6, submersed flower (06.00 h of the next morning); beginning of fruit development.

days after the opening of the first flower of an inflorescence, the first roots at the end of the branches appeared. When branches separated from the mother plant, they became new individuals (Fig. 2D).

The dehiscent fruits matured underwater 18–25 days after anthesis. Mature seeds (approximately 1 mm long) were released by explosive dehiscence of the fruits, following mechanical stimulation.

After the opening of the flowers, all staminodes and stamens were closely attached to each other, and staminodes were hiding both the stamens and the stigmas. In the absence of flower visitors, the staminodes moved outwards in a nastic movement and exposed anthers and stigmas. This movement initiated between 11.30 h and 12.00 h and maximum aperture occurred around 14.00 h ($N = 100$). In flowers which received visits, the staminodes remained in their initial position ($N = 50$).

On average, each flower produced, 480 238 (SD = 42 871; $N = 10$) small ($16 \pm 2.4 \mu\text{m}$, $N = 20$), psilate pollen grains, and 99% of the grains were viable ($N = 10$). Each carpel contained 87.3 (SD = 25.3) ovules, summing up to 349.4 ovules per flower ($N = 10$). The pollen-ovule ratio, thus, was 1375 pollen grains per ovule. The flowers produced a minute quantity of nectar that could not be measured with microcapillaries.

BREEDING SYSTEM

Hydrocleys martii is self-incompatible and its flowers depend on pollinators to set fruits. Permanently bagged flowers set no fruits and only one fruit was formed after hand self-pollination, containing only five seeds. Fruit set of hand cross-pollinated flowers

Table 1. Controlled pollination experiments in *Hydrocleys martii*

Treatment	<i>N</i>	Fruit set	Fruit set (%)	Mean number of seeds per fruit
Spontaneous self-pollination	74	0	0%	0
Hand self-pollination	34	1	3%	5
Hand cross-pollination	30	24	80%	192.5
Open pollinated flowers	55	47	85.5%	232

In the treatments, spontaneous self-pollination, hand self-pollination and hand cross-pollination, the flower buds were bagged before anthesis. Spontaneous self-pollination, flowers maintained bagged; open pollinated flowers, flowers accessible to visitors. Fruit set and mean number of seeds per fruit; Caruaru, Brejo dos Cavalos, Pernambuco, Brazil.

was similar to that of flowers accessible to pollinators (Table 1). Moreover, seed set did not differ in the number of produced fruits (mean \pm SD; control flowers mean (X) = 231.897 ± 114.20 ; hand cross-pollinated flowers $X = 192.4583 \pm 98.20$; $P = 0.16$; $t = 1.40$).

FLOWER VISITORS AND FREQUENCY OF VISITS

Seven species of bees were recorded in *H. martii* flowers from 25 ponds of 5 Brazilian states: *P. palpa-*

Table 2. Flower visitors of *Hydrocleys martii* sampled in 25 water bodies of Northeast Brazil in the states of Pernambuco, Piauí, Rio Grande do Norte, Ceará, and Sergipe

Water body	Locality	<i>Protodiscelis palpalis</i>	<i>Protodiscelis</i> sp.	<i>Plebeia flavocincta</i>	<i>Dialictus</i> sp.	<i>Augochlora</i> sp.	<i>Trigona spinipes</i>	<i>Apis mellifera</i>
1	Tapacurá	X		X		X		
2	Caruaru	X		X	X		X	
3	Caruaru	X		X	X		X	
4	Sanharó	X						
5	Sanharó	X						
6	Buique	X						
7	Pedra	X						
8	Pedra	X						
9	Pedra	X						
10	Cabrobó	X						
11	Caracol	X		X				
12	Serra Negra do Norte	X	X					X
13	Serra Negra do Norte	X	X					
14	Serra Negra do Norte	X						
15	Serra Negra do Norte	X	X					
16	Serra Negra do Norte	X						
17	Serra Negra do Norte	X						
18	Serra Negra do Norte	X						
19	Serra Negra do Norte	X						
20	Serra Negra do Norte	X						
21	Serra Negra do Norte	X						
22	Serra Negra do Norte	X						
23	Serra Negra do Norte	X						
24	Caucaia	X						
25	Canindé do São Francisco	X						

lis, *Protodiscelis* sp. (Colletidae, Paracolletinae), *T. spinipes*, *Plebeia* sp., *Apis mellifera* (Apidae), *Dialictus* sp., and *Augochlora* sp. (Halictidae) (Table 2). Females and males of the species *P. palpalis* were recorded in all localities and, in 18 (72%) ponds, they were exclusive flower visitors (Sanharó, Pedra, Buique, Cabrobó, Caucaia, and Canidé do São Francisco). In three ponds, we found a second species and, in four ponds, two to three other species (Table 2). At the Serra Negra do Norte site, we recorded seven males and four females of a yet undescribed species of *Protodiscelis*.

Each *H. martii* flower was visited an average of 37 times a day at the Caruaru site ($N = 34$ flowers; 1263 visits) and 54.8 times at the Serra Negra do Norte site ($N = 10$; 548) (Fig. 4). *Protodiscelis palpalis* individuals were by far the most frequent flower visitors at both sites, and females of this species performed more than 60% of all flower visits in Caruaru and Serra Negra do Norte

The females of *P. palpalis* visited flowers from 09.30–14.00 h. The males began visiting flowers 1 h before the females, alighting even on still closed flowers (Fig. 5). From 09.30 h onward, the males

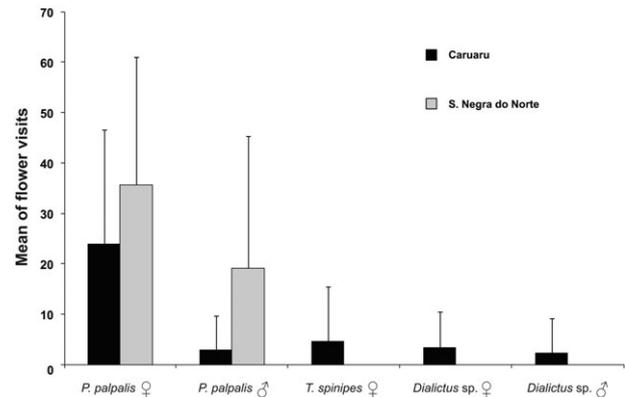


Figure 4. Average number of bee visits to single flowers of *Hydrocleys martii* at Parque João Vasconcelos Sobrinho, Caruaru, Pernambuco ($N = 34$ flowers; 1263 visits) and Serra Negra do Norte, Rio Grande do Norte ($N = 10$ flowers; 548 visits). Mean and standard deviation. *P.*, *Protodiscelis*; *T.*, *Trigona*.

patrolled and only occasionally touched the flowers. Only during the first and last hour of the anthesis of *H. martii* did the males come into contact with the cone of staminodes and took up nectar.

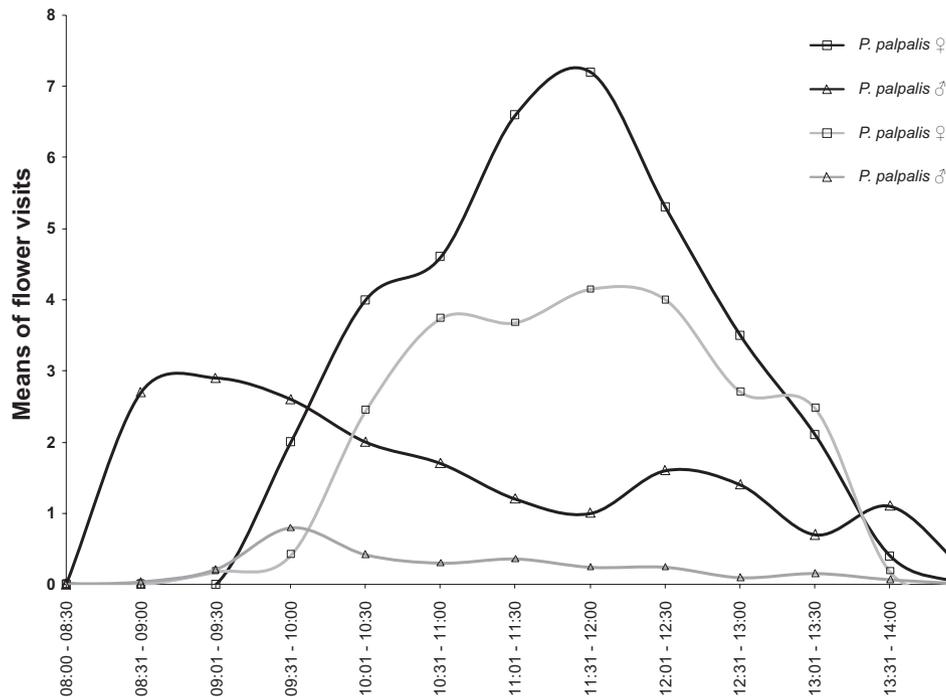


Figure 5. Means of flower visits of males and females of *Protodiscelis palpalis* to flowers of *Hydrocleys martii* along anthesis in Parque João Vasconcelos Sobrinho, Caruaru, Pernambuco ($N = 34$ flowers; light line) and Serra Negra do Norte, Rio Grande do Norte ($N = 10$ flowers; dark line).

The females of *P. palpalis* visited flowers in a unique manner compared to bees of other species. They alighted on the petals and positioned themselves perpendicularly to the orientation of the staminodes at the outside of the cone. With their head and forelegs, they pushed a few exterior staminodes outwards and advanced in the direction of rotation of the staminodal spiral along the space between the layers of neighbouring staminodes, until they reached the pollen chamber in the centre of the flower. Hidden by the staminodal cone, they remained invisible from the outside for 13.7 ± 7.1 s ($N = 131$). The duration of flower visits during the first three hours did not differ from that, from 10.00 h until the end of anthesis ($\log nX_1 = 2.57 \pm 0.52$; $\log nX_2 = 2.43 \pm 0.53$; $t = 1.09$; $P = 0.27$, X_1 , X_2 represent the mean of the transformed time into $\log n$). The bees left the flowers from the centre of the cone backwards, with the abdomen first (Fig. 6B). The pollen grains collected in the pollen chamber adhered to long plumose setae of the ventral metasomal scopa (Fig. 6D, E) and to scopal hairs with long, fine lateral branches on hind coxa, femur, and tibia (Fig. 6C).

The scopal loads of *P. palpalis* females from 25 localities contained exclusively pollen of *H. martii* ($N = 5$ females per locality). Analyses of pollen grains adhering to the body surface of males showed that 74.1% ($N = 27$) carried exclusively grains from *H.*

martii, and two males from the Caruaru site (7.4%) carried additionally a few grains of *Cuphea racemosa* (Lythraceae) and five males from Serra Negra do Norte (18.5%) also carried pollen grains of *Echinodorus* (Alismataceae) on the body surface besides pollen of *H. martii*.

None of the other bees listed in Table 2 accessed the pollen chamber of *H. martii* or contacted the stigmas. *Trigona spinipes* workers alighted on petals and staminodal cones and showed a destructive behaviour: with their mandibles, they cut the apex of the staminodes until they reached the fertile stamens. They cut stigmas, staminodes, and anthers with their mouthparts and removed the pollen grains from the anthers with their anterior legs. Foraging on flowers of *H. martii*, worker bees of *T. spinipes* visited 36–60 ($X = 42$; $N = 6$) flowers, which lasted 22–40 min ($X = 32$ min; $N = 6$) until leaving the flower patch with full corbiculae. A single flower visit lasted 10–179 s (mean \pm SD: 74 ± 97 s; $N = 46$). Bees of this species were recorded exclusively at the Caruaru site with surrounding forest cover. In such sites, 20% of the flowers were damaged by workers of *T. spinipes* ($N = 270$). Females of an unidentified species of the genus *Dialictus* gleaned the flowers, collecting the pollen grains adhering to the petals after visits of *P. palpalis*. Bees of the remaining species were only sporadic flower visitors.

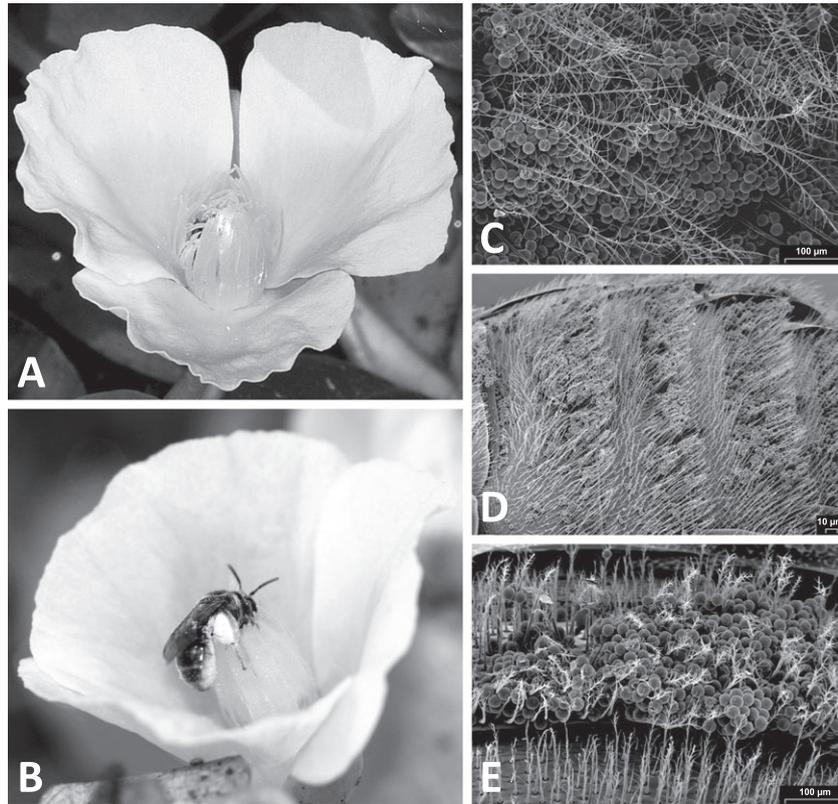


Figure 6. Flower visits of females of *Protodiscelis palpalis* to *Hydrocleys martii*, and pollen grains adhering to scopal hairs. A, female bee almost hidden within the pollen chamber. B, female leaving a flower with hind leg and metasomal scopa filled with pollen grains. The bees gain access to the pollen chamber following the space between layers of staminodes in the spiral androecium and leave the staminode cone backwards through the centre. C, D, E, pollen grains of *Hydrocleys martii* in the scopa of *Protodiscelis palpalis*. C, hind femur. D, E, ventral metasomal scopa.

Table 3. Number of pollen grains produced by flowers of *Hydrocleys martii*, attached to stigmas at the end of anthesis, remaining on petals, staminodes, and anthers, and the number of pollen grains collected by bees in Brejo dos Cavalos, Caruaru (mean, SD and %; $N = 25$)

	Number of pollen grains (mean \pm SD)	Percentage (%)
Pollen per flower	480 237 \pm 42 870	100
Pollen attached to all stigmas at end of anthesis	6 246.4 \pm 1 631	1.3
Remaining pollen grains in flowers at the end of anthesis	46 250 \pm 19 429.4	9.6
Pollen collected by flower visitors	414 558.1–453 417*	86.3–94.4

*Maximum and minimum estimate of the number of grains of pollen collected by floral visitors.

Each flower of *H. martii* contained an average of 480 000 pollen grains (Table 3). At the end of anthesis, 1.3% (approximately 6200) of the grains were deposited on the stigmas of each flower and 9.6% remained attached to the staminodes, petals, and anthers. The rest (mean of 89.1%) was collected by bees (Table 3).

Pollen grains were collected extraordinarily fast: at 10.00 h, 1 h after the opening of the flowers, approxi-

mately half of the pollen grains were already removed and, 1 h later, less than 20% of a flower's total pollen content remained (Fig. 7). Towards the end of anthesis, this number decreased slowly, reaching an average of 9.6% at flower closure. A significant difference in the number of remaining pollen grains per flower and time of anthesis was found only in the first and second hour after opening of the flowers (ANOVA: $F = 180.7099$; d.f. = 5; $P < 0.001$).

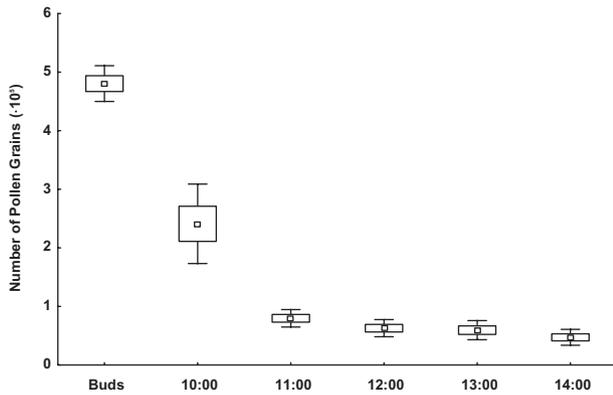


Figure 7. Number of pollen grains present in flowers of *Hydrocleys martii* during anthesis in intervals of 1 h from 08.00 h (flower buds) until 14.00 h (closing flowers) ($N = 10$ for each hour) in Brejo dos Cavalos, Caruaru, Pernambuco, Brazil (mean, SD and SE). (ANOVA: $F = 180.7099$; d.f. = 5; $P < 0.001$).

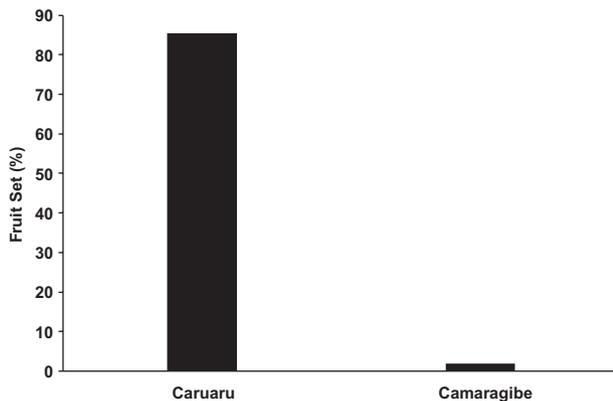


Figure 8. Fruit set of flowers of *Hydrocleys martii* accessible to flower visitors in Caruaru (natural population; $N = 55$ flowers) and in Camaragibe (Coastal Atlantic Rainforest; $N = 100$).

FRUIT AND SEED SET IN THE ABSENCE OF *P. PALPALIS*

Hydrocleys martii individuals from the Caruaru site, cultivated over 6 months at a site within secondary forest in the region of the coastal Atlantic Rainforest, where *P. palpalis* did not occur, showed an extremely low fruit set. Only two out of 100 marked flowers set fruit (2%) (Fig. 8). The fruits contained an average of 133.5 seeds, which is 57.5% of the number of seeds per fruit from the Caruaru site, where *P. palpalis* bees were abundant flower visitors of *H. martii*. At the coastal rainforest site, the flowers were visited by stingless bee workers of three species: *T. spinipes* and *Plebeia cf. minima* and *Plebeia flavocincta*, which is kept in the nearby meliponary. The visiting frequency of the stingless bees at this site was higher than that

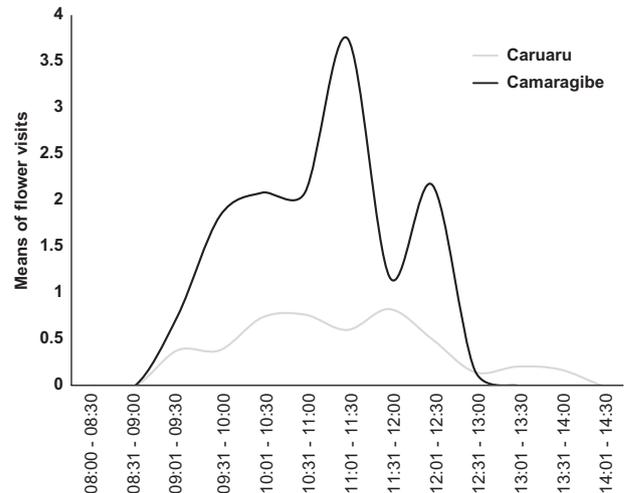


Figure 9. Mean flower visits per hour of worker bees of *Trigona spinipes* to *Hydrocleys martii* along anthesis from 08.00–14.30 h at the Caruaru site and at the coastal Atlantic Rainforest site ($N = 40$ flowers per site).

at the Caruaru site (Fig. 9). During the flower visits, the stingless bees did not access the pollen chamber.

DISCUSSION

No plant species was recognized as an important pollen and nectar resource for oligolectic *P. palpalis* other than *H. martii* at all study sites. Reciprocally, no bee species was a significant pollinator of the flowers of *H. martii* other than *P. palpalis*; at most sites, bees of this species were even the unique flower visitors. *Hydrocleys martii* and *P. palpalis*, thus, form a highly specialized association in which both partners are mutually dependent from each other.

This is unexpected because this association is characterized by temporal discontinuity and unpredictability. In the Caatinga, the aquatic, annual *Hydrocleys* plants are restricted to ephemeral ponds, which only appear during the short-lasting rainy season. In the widely predominant surrounding Caatinga-matrix, no aquatic plants occur among the xerophytic thorn-shrubs or the seasonal layer of herbaceous annuals. Moreover, rainfall in the Caatinga is highly irregular and drought years with almost no precipitation happen once or twice in a decade (Prado, 2003). Under these conditions, widely distributed polylectic pollinators of the Caatinga and generalist plant-pollinator systems would be expected to occur, as is often the case within pioneer and weedy species that colonize new environments (Baker, 1965; Waser *et al.*, 1996; Johnson & Steiner, 2000; Waser & Ollerton, 2006). Our pollination experiment conducted in the region of Atlantic Rainforest showed, however, that fruit set of *H. martii* is minute in the absence of

oligolectic *P. palpalis*. Plant and pollinator synchronize their ephemeral life activity period and reconstitute the specialized system in each isolated ephemeral pond after substantial rainfall.

Because these bees are small and might possess only low flight capacity (Greenleaf *et al.*, 2007), it is not clear how they are capable of tracking *H. martii* flowers in newly-formed ponds. We speculate that, during a possible migratory phase in the beginning of the flight season, bees may feed on other nectar sources as cited for other species of oligolectic bees (Cane & Sipes, 2006). Minckley *et al.* (1994) showed that the synchrony of phenologies between bee and host plant may be very important in the selective maintenance of specialization in *Dieunomia* (Halictidae) and argue that asynchronous females need to fly long distances to collect pollen. If such behaviour does occur in *P. palpalis*, asynchronous females could find new ponds colonized by *H. martii*. On the other hand, a high population density of *P. palpalis* at a given site may stimulate females to look for populations of *H. martii* in ponds not occupied with bees of *P. palpalis* or with a not yet saturated support capacity.

Another surprising feature of the here studied plant-pollinator system is the self-incompatibility of *H. martii* and therefore its dependency on pollinators to set fruit, comprising a highly risky breeding system for an annual plant species (Baker, 1965; Bawa, 1990). An absence or loss of the effective pollinators during a single vegetation period would compromise the survival of the whole population as a result of insufficient seed production. The specialized association, however, appears to be stable and we found no populations of *H. martii* without the associated oligolectic bee pollinators. A probably efficient dispersal of the tiny seeds, in the plumage of water birds, should favour recolonization of a temporary pond, in the case of a temporary lack of the effective pollinators.

Specialized associations of aquatic plants involving oligolectic bee pollinators in the Neotropics are also known in *Ludwigia* (Onagraceae) (Gimenes, 1991; Alves-dos-Santos, 1999), *Eichhornia* (Alves-dos-Santos & Wittmann, 1999, 2000; Alves-dos-Santos, 2002), *Pontederia* (both Pontederiaceae) (Alves-dos-Santos, 1999; Schlindwein, 2004), and *Echinodorus grandiflorus* (Vieira & Lima, 1997). By contrast to *H. martii*, however, these are long-lived plant species that occur in permanent or semi-permanent water bodies.

The staminode cone that forms the pollen chamber was the main feature that limited the access of non-specialized flower visitors to floral resources. Only female and male *P. palpalis* reached the centre of the flower following the space between spirally inserted staminodes. Females from other species were glean-

ers or pollen robbers, such as *T. spinipes*, which removed pollen grains in destructive flower visits similar to their pollen removal from tubular anthers (Buchmann, 1983). Pollen chambers which are selective for effective oligolectic bee pollinators are also known in *Opuntia* (Cactaceae) (Schlindwein & Wittmann, 1997a), *Eucnide*, and *Mentzelia* (Loasaceae) (Thompson & Ernst, 1967; Zavortink, 1972; Müller, 1995).

Because bee species other than *P. palpalis* have no access to the pollen chamber, there is no interspecific competition but harsh intraspecific competition among females of *P. palpalis* at the studied sites. During the second hour of anthesis, females had already emptied the flowers. Despite this, the high frequency of flower visits was maintained for more than 3 h. These persistent flower visits must have provided intense cross-pollen flow. Although flowers could not be emasculated without damaging and altering the flower structure to measure the deposition of exogenous conspecific pollen, the extraordinarily high stigmatic pollen loads at the end of anthesis and the high seed numbers per fruit demonstrate the efficient pollen flow between flowers of different plants in this specialized pollination system. The contribution of males of *P. palpalis* to seed set, however, was small, because they rarely visit flowers during patrol flights.

The long, branched scopal hairs on metasomal sterna and hind legs of *P. palpalis* are suited to transport the small pollen grains of *H. martii*. Females of oligolectic *Perditomorpha pampeana* and *Perditomorpha franki* (Paracolletinae) also transport small pollen grains of their host plants (Loasaceae) (Schlindwein & Wittmann, 1997a) and show scopal hairs similar to that of *P. palpalis*. Scopal hair morphology in oligolectic species is generally related to the pollen size of their host plants (Thorp, 1979). Other oligolectic species of Paracolletinae, whose host plants have very large pollen grains, show long and unbranched scopal hairs on metasomal and hind leg scopae, such as *Perditomorpha brunerii* (pollen hosts Malvaceae) (Gaglianone, 2000), *Tetraglossula bigamica* (pollen hosts *Ludwigia*, Onagraceae) (Gimenes, 1991), and *Cephalocolletes rugata* (pollen hosts *Opuntia* and *Parodia*, Cactaceae) (Schlindwein & Wittmann, 1995, 1997b).

Furthermore, females of *Protodiscelis echinodori* exhibit a scopal structure very similar to that of *P. palpalis* (A. T. Carvalho, pers. observ.). These bees are effective pollinators of aquatic *E. grandiflorus* (Alismataceae) (Vieira & Lima, 1997), which also produce tiny pollen grains. Besides *P. echinodori*, Vieira & Lima (1997) report females of several polylectic species being effective pollinators collecting pollen in *E. grandiflorus*. Staminodes and pollen

chamber are absent in the otherwise similar *Echinodorus* flowers, and bees of several species obtain free access to floral resources of *E. grandiflorus*.

The genus *Protodiscelis* Brèthes 1909 is restricted to South America and includes five described and several undescribed species (Michener, 1979, 2007; Silveira, Melo & Almeida, 2002). We recorded bees of an undescribed species of *Protodiscelis* in flowers of *Limnocharis flava* (Limnocharitaceae) also in the region of the Caatinga (A. T. Carvalho & C. Schlindwein, unpubl. data). Melo (1996) suspected that all species of the genus were oligolectic in species of Alismataceae. Ducke (1908), who described two of the species, cited '*Alisma* sp.' as food plants of both species, a genus currently considered not to occur in Brazil. Limnocharitaceae as circumscribed today (Haynes & Holm-Nielsen, 1992), together with Alismataceae, form part of a clade of basic monocotyledons. Curiously, at most study sites, *H. martii* occurred together with *Echinodorus subalatus*, flowering simultaneously in the same temporary ponds. The latter was pollinated by *Protodiscelis alismatis* (A. T. Carvalho & C. Schlindwein, unpubl. data). We recorded no host plant switches of either *P. palpalis* or *P. alismatis* from *Hydrocleys* to *Echinodorus*, or vice versa. This expresses narrow oligolecty (*sensu* Cane & Sipes, 2006) and high flower fidelity for both bee species.

This is the first pollination study representing the family Limnocharitaceae. Because of the close relationship of both partners demonstrated in the present study, as well as hints of the tight associations of other species of *Protodiscelis* with species of Alismatales, a comparative study on host-plant relationships in *Protodiscelis* and the possible reproductive interdependency of the partners could provide insights regarding their evolutionary history.

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