Floral biology and nectar production dynamics of *Pachira aquatica* Aubl. (Malvaceae) in the eastern Amazon

Biologia floral e dinâmica da produção de néctar de *Pachira aquatica* Aubl. (Malvaceae) na Amazônia Oriental

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Abstract: *Pachira aquatica* is a Neotropical tree of economic importance for local communities, although little is known about its flower biology especially flower rewards. This study aimed to analyze some aspects of flower biology of this species, focusing on nectar production dynamics. We measured nectar production and analyzed the effect of nectar removal and cross-pollination on sugar production and standing crop (the amount of nectar present in a flower exposed to pollination at a given moment). We found that *P. aquatica* has large, brush-type, perfect flowers, with white colored petals, red stamens and a strong floral scent. Flowers are protandrous and dichogamous. Pollen viability is about 96%. Anthesis is nocturnal and asynchronous. The nectar is relatively abundant (99.8 ± 118.5 μL) and diluted (15.2 ± 4.7% p/p) and its production increases in the morning period. Neither nectar removal nor cross-pollination affected sugar production. Standing crop was not significantly different from the control, indicating that there is probably a low visitation rate. Both flower and nectar production characteristics indicate that this species has the potential to be visited by different flower visitors, especially bats, birds and bees.

Keywords: Nectar removal effects. Sugar production. Standing crop. Cross-pollination.

Resumo: *Pachira aquatica* é uma árvore neotropical de importância econômica para as comunidades locais, contudo pouco se conhece sobre sua biologia floral, principalmente sobre as recompensas florais. Este estudo pretende analisar alguns aspectos da biologia floral, detalhando a dinâmica de produção de néctar. Foi construído o perfil de sua produção pela flor durante 12 h. Analisou-se o efeito da remoção de néctar e da polinização cruzada sobre a produção de açúcar, e também o *standing crop*. As flores de *P. aquatica* são grandes, tipo pincel, perfeitas, odoríferas, com pétalas brancas e estames vermelhos. Apresentam dicogamia protândrica e viabilidade polínica de 96%; antese é noturna e assincrônica. O néctar é relativamente abundante (99,8 ± 118,5 μL) e diluído (15,2 ± 4,7% p/p), aumentando no período da manhã. A sua remoção tende a afetar a produção de açúcar, apesar de a relação não ser significativa. Não se verificou efeito da polinização sobre a sua produção. O *standing crop* não foi significativamente diferente do controle, indicando possível baixa taxa de visitação. As características florais e de produção de néctar indicam que a planta tem potencial de atrair uma ampla gama de visitantes florais, especialmente morcegos, aves e abelhas.

Palavras-chave: Efeito da remoção de néctar. Produção de açúcar. *Standing crop*. Polinização cruzada.

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INTRODUCTION

Floral biology involves many physiological, morphological, ecological and evolutionary aspects of flowers that permit, among many other things, an understanding of the reproductive biology of the dominant plants on earth – the angiosperms (Dafni *et al*., 2005). For animal-pollinated plants in particular, it is crucial to study flower attraction, that is, floral display and rewards to pollinators, in order to comprehend plant-pollinator relationships and therefore, angiosperms reproductive strategies (Endress, 1994).

Nectar, a source of carbohydrates for flower visitors, is the most common plant reward for pollinators, and an important source of energy (Simpson & Neff, 1983; Proctor *et al*., 1996). Nectar shows variability in its production within and among plants, that depends on internal (genetic or morphological) and environmental factors (Pacini & Nepi, 2007). It is secreted in particular rhythms and can be reabsorbed over the life of the flower (Galetto & Bernardello, 2005). Its production dynamics are defined by the temporal patterns of secretion, cessation, and reabsorption (Galetto & Bernardello, 2005; Pacini & Nepi, 2007) that are linked to environmental conditions and pollinator visitation (Galetto & Bernardello, 2005). Understanding nectar secretion patterns and some floral biology aspects related to this pattern, such as morphology, sexual allocation and floral display, will give us insights into the attractiveness of plants to pollinators and the potential characteristics of these pollen vectors and plant fitness.

Pachira aquatica Aubl. is native to South and Central America where it is found mostly in riparian forests along riverbanks (Lorenzi, 2002). The species can grow very well in dry soils in the Brazilian biomes (Peixoto & Escudeiro, 2002; Silva, 2011) and is widely used in many Brazilian cities as an ornamental (Peixoto & Escudeiro, 2002; Silva *et al*., 2012). Its seeds are edible and highly appreciated by indigenous Amazonian populations (Correa, 1984). Despite its importance for local populations, little is known about the floral biology and there is no available information about nectar dynamics of this species or the genus.

This study aimed to understand flower biology aspects, such as flower attractiveness, time of anthesis, and nectar secretion patterns to answer the following questions: 1) nectar secretion often increases during the flower life span but can also show patterns of reabsorption (Burquez & Corbet, 1991), so what is the nectar secretion pattern in *P. aquatica*?; 2) does nectar removal by pollinators have an effect on nectar production (positive or negative) or have no effect on solute production?; 3) does nectar secretion stop or decrease after pollination to save plant resources?; 4) given that differences in nectar solute between of bagged flowers (covered with pollination bags in which nectar cannot be removed by visitors) and randomly-opened flowers could indicate effective visitation from pollinators, so are there differences between nectar standing crop and nectar from bagged flowers?

MATERIALS AND METHODS

The study was conducted in the Caxiuanã National Forest (at the Ferreira Pena Research Station), in Pará state, Brazil, from 18 to 23 September, 2011. Caxiuanã occupies an area of 324,060 hectares near Caxiuanã Bay between the cities of Portel and Melgaço. The region has a wide array of natural ecosystems of the Amazon region, such as upland forest, floodplain and igapó (Lisboa, 2009).

FLORAL BIOLOGY

A morphological analysis was performed with fresh flowers (n = 10) using a digital caliper. Flowers were collected from ten individuals growing on the riverbanks. We took *in situ* observations using a speedboat totaling approximately 80 hours of observation. Stigmatic receptivity, anther dehiscence and release of pollen grains were recorded, as well as the periods of pre-anthesis, anthesis and post-anthesis.

In ten flowers, stigmatic surface was characterized morphologically and physiologically according to Heslop-Harrison & Shivanna (1977). We used hydrogen peroxide (H_2O_2) to determine the receptivity of the stigma (Dafni *et al*., 2005). We also counted the number of pollen grains present on the stigma of five flowers belonging to different plants.

We tested the occurrence of lipid secretion on the exine of pollen grains with distilled water and verified under an optical microscope and stereomicroscope (Dafni *et al.*, 2005). We stained pollen grains from five anthers of different flowers with acetocarmine staining solution to measure pollen viability (Alexander, 1980). We counted 200 grains per anther.

Floral odors were examined by dissecting the floral whorls and placing them in different plastic containers. They were kept closed for about ten minutes and we then verified odor emissions by these structures. The presence of fragrance glands was tested using neutral red solution in five newly open flowers. After 15 minutes, the flowers were washed in distilled water and checked for the presence of stained osmophores or fragrance glands (Vogel, 1983).

NECTAR PRODUCTION DYNAMICS

Nectar was extracted with graduated capillary glass tubes without removing the flowers from the plant, except for the flowers measured at the last sample time (12:00) that were removed and then measured. We measured volume through graduated capillary tubes and sugar concentration weight/weight (w/w) with a pocket refractometer. The amount of sugar produced was expressed in mg following the methods of Galetto & Bernardello (2005).

SECRETION AND REPEATED SAMPLING

The experiment was conducted over two nights. Flowers were bagged in pre-anthesis the evening before sampling and sampled in sets containing six flower from different plants (exception n = 4 for set 2, where we lost two flowers) (Table 1). Set 1 was sampled at 00:00, then resampled 04:00, 08:00 and 12:00. Set 2 was first sampled at 04:00, then again at 08:00 and 12:00. Set 3 was sampled first at 08:00 then again at 12:00 and Set 4 (control) was only sampled at 12:00 (Table 1).

Table 1. Experimental design of nectar removal for each flower set and the times that removals were performed. Each flower set was sampled at different times. The removals marked with X in bold were 'virgin flowers' not sampled before, these measurements were used to build the nectar profile.

Sets of flowers	Time of nectar removal from flowers			
	00:00	04:00	08:00	12:00
Set 1 (n = 6)	**X**	X	X	X
Set 2 (n = 4)		**X**	X	X
Set 3 (n = 6)			**X**	X
Set 4 (n = 6)				**X**

NECTAR PROFILE

The nectar profile was created using the flowers that were not previously sampled or 'virgin' at each time interval (00:00, 04:00, 08:00 and 12:00) from the sets described above (Table 1). We calculated nectar volume, concentration and the amount of sugar and plotted three curves: nectar volume *versus* time intervals; sugar concentration on nectar *versus* time intervals and the amount of sugar production *versus* time intervals.

REMOVAL EFFECT (POLLINATOR EFFECT) ON NECTAR PRODUCTION

To test if nectar removal affected nectar production, we calculated the amount of sugar produced by flowers that were sampled (depleted of nectar) at different times (Table 1): once (Set 4), two (Set 3), three (Set 2) and four times (Set 1). Nectar depletion simulates floral visitors and/or pollinators activity. As numbers were not completely even between sets and the data were not normal (Shapiro-Wilk) a Kruskal-Wallis one-way analysis of variance was used to test for differences between flowers, which had been sampled in the four sets at 95% significance level.

CROSS POLLINATION AND ITS EFFECT ON NECTAR SECRETION

Three different flowers a night for three nights were selected randomly, totaling nine flowers. At 00:00 nectar was collected and the stigma extensively pollinated with outcross pollen (from two to three pollen donors) to simulate a pollinator. Flowers were then bagged and left until 04:00 when nectar was collected again.

To identify whether pollination had an effect on nectar production, we plotted the amount of sugar collected before and after pollination and compared it with the amount of sugar collected from the nectar removal study, from the same time interval (the same treatment of nectar removal without the cross pollination). Then we used Mann-Whitney test for non-parametric data to compare the amount of sugar between treatments, that is, the amount of sugar collected at 00:00 *versus* 04:00 in pollinated flowers and amount of sugar collected at 00:00 *versus* 04:00 in non-pollinated flowers.

STANDING CROP

Over the three nights, nine flowers (per experiment) that had not been previously bagged (open to pollinators) were sampled for nectar at four sample times of 00:00, 04:00, 08:00, and 12:00. This was to estimate 'standing nectar crop' or how much nectar is available for visitors at any given time. Conversely, this can also be an estimate of when nectar visitors are active by comparing the standing crop values to those of the bagged nectar removal study. To compare nectar solute between open and bagged flowers (controls) at each sample time we used the Mann-Whitney test for non-parametric data.

RESULTS AND DISCUSSION

FLORAL BIOLOGY

Pachira aquatica has morphologically perfect flowers, they are large, solitary, pedicellate and placed at the apex of the branches. Flowers are about 215 mm long and 246 mm in diameter (Table 2). Each flowering branch has many developing buds but only one flower opens per day. According to Ferreira *et al*. (2005) the plant flowers throughout the year, with a few open flowers per night. The corolla is pentamerous, diapetalous, actinomorphic, with a fleshy consistency. It is white and smooth on the adaxial surface and has many trichomes on the abaxial surface. It is a brush shaped flower, with many stamens, which act as the attractive unit.

Table 2. Comparative data (mean, minimum and maximum) of flower and floral structures size (mm) of *Pachira aquatica* Aubl. in Caxiuanã, Pará, Brazil (n = 10).

Variable (n = 10)	Mean	Minimum	Maximum
Flower long (mm)	214.9	150	332.4
Flower width (mm)	246.1	180	277.7
Peduncule (mm)	27.5	6.7	57.1
Petal (mm)	271.4	229.3	317.2
Number of petals	5	5	5
Calyx (mm)	22.1	10.6	29.8
Number of stamens	207.8	130	292
Major filament (mm)	123.5	112	137
Minor filament (mm)	177.1	153	196
Filament tube (mm)	61.4	43	82.7
Ovary width (mm)	6.4	5	8
Ovary long (mm)	6.2	5	7.5
Style (mm)	240	201	292
Stigmatic surface (mm)	4.0	3.6	4.6
Stigmatic lobules (mm)	5	5	5
Locules number	5	5	5
Carpels number	5	5	5
Ovules number	50	35	50

The calyx base is green, with many white trichomes on the inner surface (nectary). According to Fahn (2000) and Rocha *et al*. (2010) the nectar of the family Malvaceae is usually secreted on the inner surface of the connate sepals. Studies conducted by Machado & Sazima (2008) in *Melochia tomentosa* L. characterized trichomatic nectaries, located on the inner surface of the sepals, between the calyx and the basal part of the corolla tube, where there are small spaces where nectar can accumulate, as is the case in *Pachira aquatica*.

On the external surface of the calyx there are about five glands (n = 10), which were already present in the bud stage. Many ants apparently from the same species can be observed visiting these glands (Figure 1E-1F) in all stages of flower development, including the bud stage, and during day and night. These ants could potentially protect the plant and especially the flower from insect herbivory (Bentley, 1976).

The androecium consists of stamens of different sizes (Table 1). Filaments are red in color from the apical portion to the middle, and white from the middle region to the basal portion. The anthers are dorsifixed, monadelphous with longitudinal dehiscence. The anthers are versatile, so that the position of the aperture can be extrorse or introrse from the center of the flower. The stamens are heterodyne, the basal portion being connate into a tube (61.4 mm) and the free portion of the stamen filaments ranging in size from 123 to 177 mm. On average there were 207 stamens per flower (n = 10, Table 1).

Pollen grains have pollenkitt, meaning that many grains of pollen clump together which helps to grip the body of the pollinator. When the anthers are dehiscent, we saw pollen grains in different whorls of the flower, in the style, filaments and petals, which may characterize a possible secondary exposure of the pollen grains, as it is seen in other species of the Malvaceae family (Walker-Larsen & Harder, 2000; Rangel *et al*., 2011).

The viability of pollen grains was 96% (n = 5). We observed many tufts of black hairs in the anthers of flowers of *P. aquatica*, probably belonging to bats, but we could not confirm it.

The test to detect the presence of odor using sealed containers was positive for the petals and androecium, where the latter had the most intense odor. The neutral red test was positive just for the petals, but neither the androecium nor the anthers stained, so it was not possible to confirm the result.

The gynoecium has a white, superior ovary, which is gamocarpelar and pentacarpelar with 5 *loculi* and about 50 eggs per flower (n = 10). The style is terminal, red and white, with white trichomes near the ovary. The styles is long (240 mm) and longer than the stamens, sustaining the flowers herkogamy (spatial separation of sexual functions in flowers).

The stigma is papillose, wet and has five lobes. The stigmatic surface is large with varying size in the pre-anthesis, anthesis and post-anthesis. The lobes are joined at the apex in the bud (1.5 to 1.9 mm) and the lobes are expanded in the flower (3.57 to 4.56 mm). Post-anthesis stigmas are similar to the bud, however, darkened and not receptive. The number of pollen grains on stigmatic surface ranged from 153 to 445 (x = 275, n = 5), indicating pollen deposition and maybe pollination. To date, we do not know if *P. aquatica* is self-compatible, but we know that other species from the genus, *Pachira quinata* (Jacq.) W.S. Alverson are not selfing in natural populations but have a "cryptic self-incompatibility system" that allows its reproduction when pollen from other individuals is scare (Fuchs *et al*., 2003). It is important to perform studies to identify the species sexual system.

FLORAL OPENING

Flower anthesis of *P. aquatica* is nocturnal and anther dehiscence occurs in the bud pre-flower anthesis at about 18:00 when buds are 200 mm long approximately (n = 5), indicating protandry. At this stage, the stigma lobes have just begun to open, with the stigmatic surface expanded only in the fully open flowers. The flowers of different species of Malvaceae also exhibit protandry, with pollen being viable and available during the bud phase (Rangel *et al*., 2011) keeping their viability until the flower stage (Gaglione, 2000). Gaglione (2000) studied the floral biology of 17 species of Malvaceae belonging to the genera *Sida*, *Sidastrum*, *Malvastrum*, *Gaya*, *Wissadula* and *Urena*; sixteen of these species were protandrous and their stigma expanded only after full flower opening as observed at *P. aquatica*.

In *Pachira aquatica* the flowers opened asynchronously, and at different times, running from 20:00 to 02:00 (Figures 1A-1D). The newly opened flowers have an intense odor during the night (sweet and floral). At this stage, the stamens and the style are erect and deflexed and the petals are curved. The presence of many red and white stamens stand out from the foliage. In senescent flowers (Figure 1D) petals, sepals, androecium and gynoecium are persistent, but brown in color later in the day. At this stage, the stigma is closed, no longer receptive and anthers have few or no pollen grains available.

Figure 1. A) Flower bud of *Pachira aquatica* Aubl. prior to opening; B) newly opened flower at night; C) open flower at day and D) senescence flower; E-F) flower buds of *P. aquatica* Aubl. Note the presence of nectariferous glands on the base of the calyx (black arrows) and the presence of ants.

NECTAR PRODUCTION DYNAMICS

Nectar profile

On average, bagged (control) flowers produced 17.5 ± 23.3 mg of sugar, and 99.8 ± 118.5 μL of nectar at a concentration of 15.2 ± 4.7% w/w. After midnight until morning (from 00:00 to 08:00) there was little or no increase in nectar volume and sugar production (Figures 2A, 2C). After 08:00 there was an increase in both parameters. Concentration seems to decrease until 04:00 and increase in the morning (after 08:00) (Figure 2B). The latter indicates that nectar production is slower at night and probably shows a period of cessation between 04:00 to 08:00. Nectar production increases considerably in the morning (Figure 2).

Nectar removal effects

There was no significant difference between the total amount of sugar production in flowers sampled 1, 2, 3 and 4 times throughout the night (H = 3.077; p-value = 0.3799), although the mean amount of sugar is much higher for the control (sampled only once, Figure 3). This result indicates that flowers show a trend to reduce nectar production when they are sampled previously or maybe when they are visited. Bobrowiec & Oliveira (2012) studied the effect of nectar removal in four bat-pollinated species of the Brazilian Cerrado and found that most of them (*Bauhinia holophylla*, *Hymenaea stigonocarpa*, *Luehea grandiflora*) showed an increase in nectar production with nectar removal, except for one species (*Caryocar brasiliense*) that showed no significant effect. Galetto & Bernardello (2004) showed that nectar removal could have a positive, neutral or negative effect on sugar production in different species from the same genus, in this case *Ipomoea* (Convolvulaceae), showing there is great variability in this respect. Nevertheless, in certain environmental conditions, it could be advantageous to flowers to detect the amount of nectar and replenish it, favoring flower visitation. This strategy could save

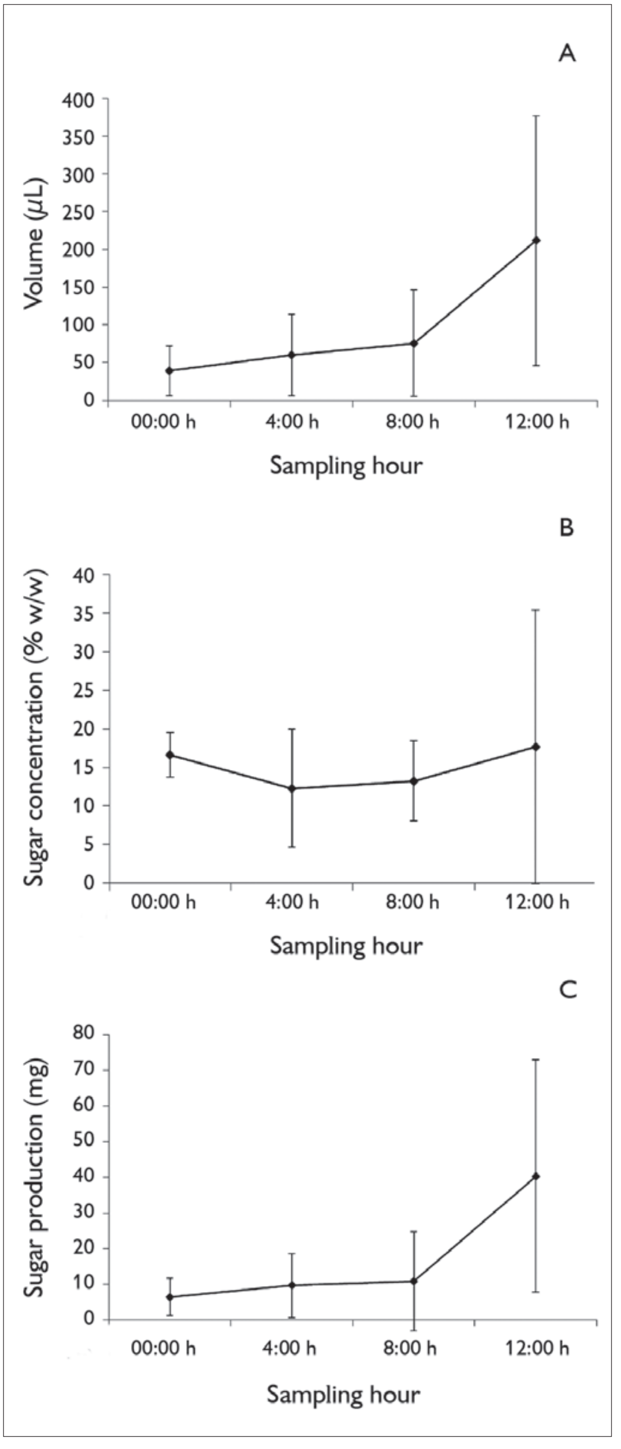

Figure 2. Nectar production profile of *Pachira aquatica* Aubl. over the flower life span in the Amazonian forest of Caxiuanã, Pará, Brazil. A) Nectar volume; B) nectar sugar concentration; C) milligrams of nectar sugars. Bars represent standard deviation.

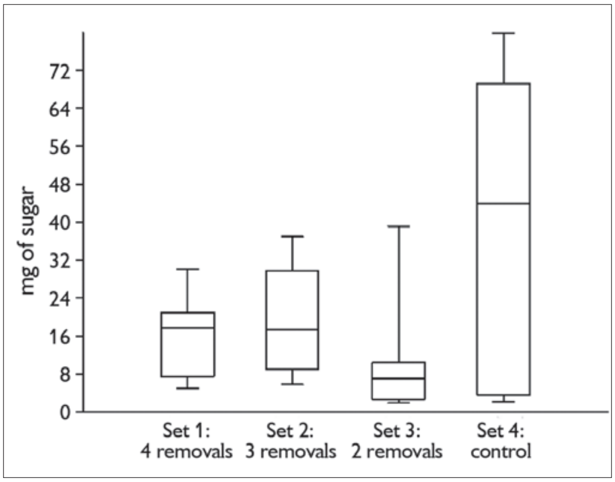

Figure 3. Box plot for nectar removal effects in the different sets of flowers. Set 1: four removals at 00:00, 04:00, 08:00 and 12:00; Set 2: three removals at 04:00, 08:00 and 12:00; Set 3: two removals at 08:00 and 12:00; Set 4: control, just one removal at 12:00.

flower resources (Castellanos *et al*., 2002) and could be advantageous in water-stressed environments such as the Cerrado. The Amazon region does not suffer water-stress comparable with that of the Cerrado, so, it is not relevant for plants to retain water resources to the same extent and we could find different responses to nectar removal in bat pollinated flower in this region.

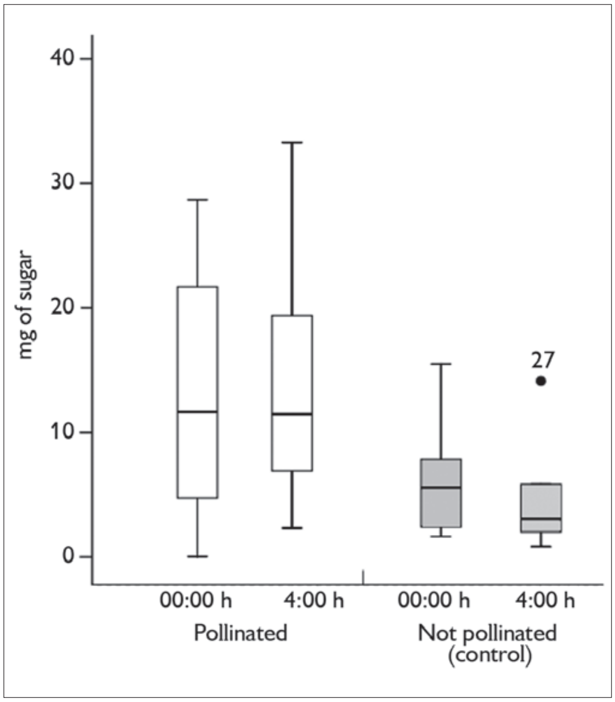

Figure 4. Box plot for the effect of pollination on sugar production compared to no pollinated flower, in both treatments the nectar was removed two times (in 00:00 and 04:00 h), but in white boxes the flowers were cross pollinated at 00:00, and in grey boxes they were not; the black point is an outlier. The tendencies are similar in both treatments, the solute before and after removals remains almost the same in pollinated ($T = 31.5$, $p = 0.45$, $n = 9$) and no pollinated ($T = 12$, $p = 0.38$, $n = 6$) flowers. Therefore, pollination does not affect sugar production.

Pollination effect

The amount of sugar produced was not significantly different after pollination ($T = 31.5$, $p = 0.45$, $n = 9$), as was the control ($T = 12$, $p = 0.38$, $n = 6$; Figure 4). Results indicate there is not a pollination effect on sugar production, at least for this short interval. The flowers were left for a four-hour interval due to logistics of taking measurements at night, however the style is relatively long (about 240 mm) and the plant response could be developed just after pollen tubes have reached a certain length. Some plant species produces post-pollination nectar that is advantageous to the plant because it maintains the attention of generalist pollinators in the species (Harder & Barrett, 1992). Nevertheless, it is necessary to perform pollen viability and pollen tube growth studies to verify if our time interval (4 hours) was appropriate to capture a pollination effect. Interestingly, flowers used to assess pollination effect on sugar production produced larger amounts of sugar than the control (Figure 4), indicating a high variability between individuals and flowers.

Standing crop

The amount of sugar produced between the flowers that were bagged (not available to pollinators) and the standing crop of open flowers were not shown to be significantly different, apart from the first measurement time – 00:00 h (Figure 5; 00:00 p-value = 0.016, 04:00 p-value = 0.700, 08:00 p-value = 0.517, and 12:00 p-value = 0.141).

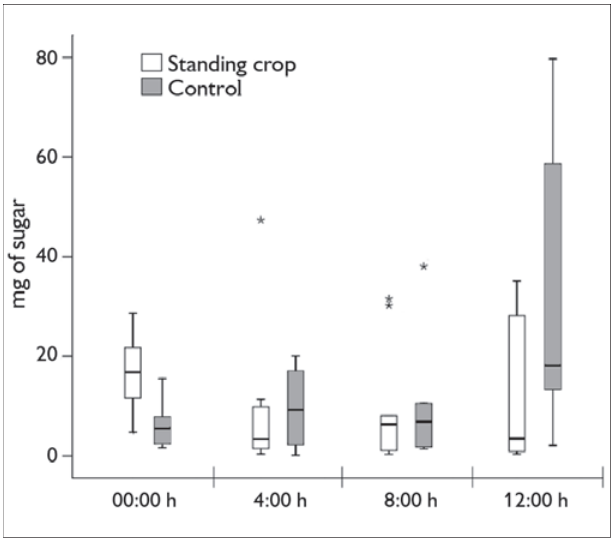

Figure 5. Box plot for standing crop measurements at different times and the control treatment. Only the first treatment (00:00 h) exhibited a significant difference. Stars represent outliers.

At 00:00 the standing crop amount of sugar was higher than the control (bagged flower) contrary to our expectations, given the results of the negative removal effects on solute production. The latter indicates that it could be due to chance, experimental error and/or high variability in nectar production between flowers. It is curious that the control showed more variability than nectar standing crop, it would be expected that the latter would be more variable (Galetto & Bernardello, 2005) given its exposure to flower visitors which could extract variables amounts of nectar or not. This could be the result of natural variability of this species flowers, sampling error, because the open flowers were better sampled for nectar (they could be removed from the plant and taken back to the lab), whereas the nectar from the bagged flowers had to be taken with the flowers still on the plant, without damaging the flowers. Another possibility is an effect of the bag on nectar production.

Pachira aquatica exhibits attractive flowers to a wide variety of animal visitors; bees and birds have been observed visiting the tree by day and bats at night (Peixoto & Escudeiro, 2002; Ferreira *et al.*, 2005). A flower characteristic that make this species attractive to bats is nocturnal anthesis, strong scent, relatively large amounts of nectar at a medium concentration, white color that is highly visible at night and large flowers (Proctor *et al.*, 1996). According to Ribeiro *et al.* (1999) members of the Malvaceae family include many bat-pollinated species, in such cases the flowers are 'brush' shaped with large amounts of pollen. During field surveys, we observed bats swooping over the plants of *P. aquatica* at night and some bees collecting pollen from the anthers during the day. *P. aquatica* also shows floral characteristics attractive to diurnal pollinators such as birds that could be attracted by the relatively high amounts of nectar present during the morning period, red and white corolla parts and tubular shape (Proctor *et al.*, 1996). Pollen could be the main reward for many bee species, which (in contrast to birds and bats) are not big enough to reach the nectaries, unless they are long-tongued bees which could access this resource.

Given many references in the literature indicating that the flowers of this species are visited by bats, requiring large amounts of nectar, why do we have such a reduced production at night? Potentially, production is less at night compared to in the morning such that the flower could accumulate volumes at night that reach 150 μL and attractive to bats (Nicolson, 2007). Providing small amounts of nectar drives pollinators to forage actively on different flowers (whereby pollinating them) until they visit enough flowers to satisfy their energetic requirements (Proctor *et al.*, 1996).

CONCLUSIONS

The flowers of *P. aquatica* are perfect, herkogamous, protrandrous, nectariferous, and odouriferous, with nocturnal and diurnal anthesis. In general, nectar dynamics showed that nectar production is very variable between flowers and it is not influenced by nectar removal or cross-pollination. The flowers exhibit morphological, phenological and nectar production characteristics that potentially attract different flower visitors and pollinators guilds, especially bats. The nocturnal and diurnal anthesis along with flower displacement of *P. aquatica* may be a strategy to assure pollination (Primack, 1985; Sazima *et al*., 1994). Thus, if bats do not pollinate them at night, there is a greater chance that they maybe pollinated by diurnal pollinators such as birds and bees.

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