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how light intensity affects foraging activity  
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a atividade de forrageio no cambuci, uma Myrtaceae neotropical

*Rodolfo Liporoni Dias*



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Universidade de São Paulo

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Orientador(a)

*Dedico este trabalho ao meus pais, Mara e Nei,  
e a todos os colegas que adoeceram física  
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## Abstract

The foraging activity of day-active bees often rely on flower availability, light intensity and temperature. However, some bees developed a nocturnal habit and fly during the dusk, dawn and night. We still do not know how these bees cope with environmental factors, especially dimmer light levels, to explore flower earlier than their diurnal relatives. Given that typical bee apposition compound eyes work better in brighter environments and function in their limits in dim-light conditions, we can expect a larger dependence of light intensity for nocturnal groups. Nocturnal and crepuscular bees are frequent visitors of cambuci (*Campomanesia phaea*, Myrtaceae) in southeastern Brazil. We aimed to investigate how light intensity affects flower visitation of cambuci by nocturnal and crepuscular bees, also controlling for other environmental factors. We counted visits per minute along 30 nights/twilight in 33 cambuci trees from a commercial orchard, measuring the following environmental variables: light intensity, flower availability, temperature, air relative humidity and wind speed. Light intensity is the only variable that explained flower visitation of nocturnal bees in cambuci, which peaks at intermediate light levels that occur around 30 minutes before sunrise. The minimum light intensity threshold to bees start flying is of  $0.00024 \text{ cd/m}^2$ , the first recorded value for nocturnal and crepuscular bees finding flowers in an agro-forest context. Our results highlight for the first time how nocturnal bees rely on light to explore resources and show that the bees light-dependent foraging activity is not always linear, as postulated by previous theoretic models. This is the first step to understand how nocturnal bees react to environmental factors. Our findings also bring concerns about possible negative effects of light pollution at night for cambuci-crepuscular bees interaction.

**Key-words:** sensory ecology; *Ptiloglossa*; *Megalopta*; *Megommation*; *Zikanapis*; nocturnal insects; Atlantic forest; crepuscular habit; solitary bees.

## Resumo

O forrageio de abelhas geralmente depende de disponibilidade de flores, intensidade de luz e temperatura. Contudo, algumas abelhas desenvolveram hábitos noturnos e voam durante os crepúsculos e a noite. Não se sabe como essas abelhas lidam com os fatores ambientais, especialmente os reduzidos níveis de luminosidade. Dado que os olhos compostos de aposição de abelhas funcionam melhor em maiores luminosidades, espera-se uma maior dependência da luz para os grupos noturnos. Abelhas noturnas e crepusculares são visitantes frequentes do cambuci (*Campomanesia phaea*, Myrtaceae) no sudeste do Brasil. Nosso objetivo foi investigar como a intensidade de luz afeta a visitação floral do cambuci por abelhas noturnas e crepusculares, controlada também por outros fatores ambientais. Para isso, contamos as visitas a cada minuto ao longo de 30 noites/crepúsculos em 33 árvores de cambuci em um pomar comercial, medindo as seguintes variáveis ambientais: intensidade de luz, disponibilidade de flores, temperatura, umidade e velocidade do vento. A intensidade de luz foi a única variável que explicou a visitação floral de abelhas noturnas no cambuci, a qual tem um pico em níveis intermediários de luz que ocorrem ao redor de 30 minutos antes do nascer do sol. O limiar mínimo de intensidade de luz para as abelhas começarem a voar foi de  $0.00024 \text{ cd/m}^2$ , o primeiro valor registrado para abelhas noturnas e crepusculares procurando por flores em um contexto agroflorestral. Nossos resultados destacam pela primeira vez como as abelhas noturnas dependem da luz para explorar recursos e mostram que essa dependência, para abelhas em geral, não é sempre linear, como postulado por modelos teóricos prévios. Este é o primeiro passo para entender como abelhas noturnas reagem a fatores ambientais. Nossos dados também trazem alertas para possíveis efeitos negativos da poluição luminosa à noite para a interação entre cambuci e abelhas noturnas.

**Palavras-chave:** ecologia sensorial; *Ptiloglossa*; *Megalopta*; *Megommation*; *Zikanapis*; insetos noturnos; Mata Atlântica; hábito crepuscular; abelha solitária.

## Introduction

Most bees are active during the day, especially under sunny and clear skies (Michener 2007). Surprisingly, however, nocturnal bees do exist and forage in search for flowers during the low light intensities of the night, between sunset and sunrise (Warrant 2007). These bees include two groups: crepuscular and truly nocturnal. Crepuscular bees fly during dawn or dusk or both, while truly nocturnal bees fly along all night or for the most part of it. Approximately 1% of all known bee species – *ca.* 250 – are nocturnal, and they are distributed among four families: Andrenidae, Apidae, Colletidae and Halictidae (Burgett and Sukumalanand 2000, Wcislo and Tierney 2009).

Mainly because of historical biases for studies with diurnal groups, we still know little about the basic biology of most nocturnal bees taxa, especially about their distribution, behavior and interactions with plants (Michener 2007, Carvalho *et al.* 2012). Bee species in the exclusively nocturnal genus *Megalopta* (Halictidae) are among the most studied. This genus has undergone an extensive radiation, reaching the current 30 species, and is distributed along the Neotropical region, from South Mexico to the north of Argentina, especially in Brazil (Engel 2000, Wcislo *et al.* 2004, Santos and Melo 2015). Other important group is the subfamily Diphaglossinae (Colletidae), which is also restricted to the Americas and contains almost 100 dim-light foraging species, mostly within genera *Caupolicana* and *Ptiloglossa* (Silveira *et al.* 2002, Wcislo and Tierney 2009). Our current knowledge is restricted to some information about nesting behavior (Janzen 1968, Roberts 1971, Santos *et al.* 2010, Sarzetti *et al.* 2013), abundance and seasonality (Wolda and Roubik 1986), nocturnal activity hours (Linsley and Cazier 1970, Roulston 1997) and feeding resources (Wcislo *et al.* 2004, Smith *et al.* 2012, Sarzetti *et al.* 2013) of some species, especially of genera *Megalopta* (Halictidae) and *Ptiloglossa* (Colletidae). Many questions related to basic biology remain open. For instance, we still do not know what environmental factors affect nocturnal bees activity.

Usually, the main factors controlling bee foraging activities are flower availability, temperature and light intensity, with relative humidity and wind speed being secondary (Dafni *et al.* 2005). First of all, flower abundance can positively affect the number of bees visiting a plant and their flower visitation rate (*e.g.*, Somanathan and Borges 2001). Second, given that bees need to reach a minimum thoracic temperature to be able to fly (Contrera and Nieh 2007) and then avoid overheating during flight, foraging activity is determined by thermal constraints (Willmer 1983). Finally, different luminosity levels seem to be important to trigger the first flights in several solitary and social bee species (Linsley 1958, Figueiredo-Mecca *et al.* 2013, Streinzer *et al.* 2016). The relationship among these factors is generally complex and the most important one is context-dependent (Linsley 1958, 1978). For instance, social bees such as Neotropical Meliponini depend both on a minimum light intensity level and a warm temperature, usually reached during sunrise, to start their foraging activity (Figueiredo-Mecca *et al.* 2013, Streinzer *et al.* 2016). Nocturnal bees, however, typically fly in lower temperatures and luminosities (Wcislo and Tierney 2009). In such conditions, nocturnal animals usually depend on more sophisticated thermoregulatory capabilities (Willmer and Stone 1997) and we do not know if these bees can thermoregulate. If nocturnal bees could actively warm up their thoracic temperature prior to fly in some way, temperature probably would not be a limiting factor over activity.

Navigation in dim light seems to be the major challenge bees face to forage at night (Warrant 2008). Light levels during the night are reduced by a factor of  $10^8$  in relation to daytime, and all bees present apposition compound eyes that work better in bright environments (Warrant 1999). Nocturnal insects, such as moths and beetles, have superposition compound eyes that catch light from several directions and focus it in a single photoreceptor to increase sensibility in dim light. In typical apposition compound eyes, such as found in bees, each ommatidium catches light from a single direction and demands that enough light is coming to

form a proper image. Thus, as light intensity decreases after sunset, bees' apposition compound eyes have lower photons to catch and function in their limits (Warrant *et al.* 2004, Warrant 2008, Warrant and Dacke 2011). This photic condition change between daytime and nighttime represented the invasion of a new and extreme environment for bees (Warrant and Dacke 2011) and, therefore, light intensity could play an important role in nocturnal bees activity.

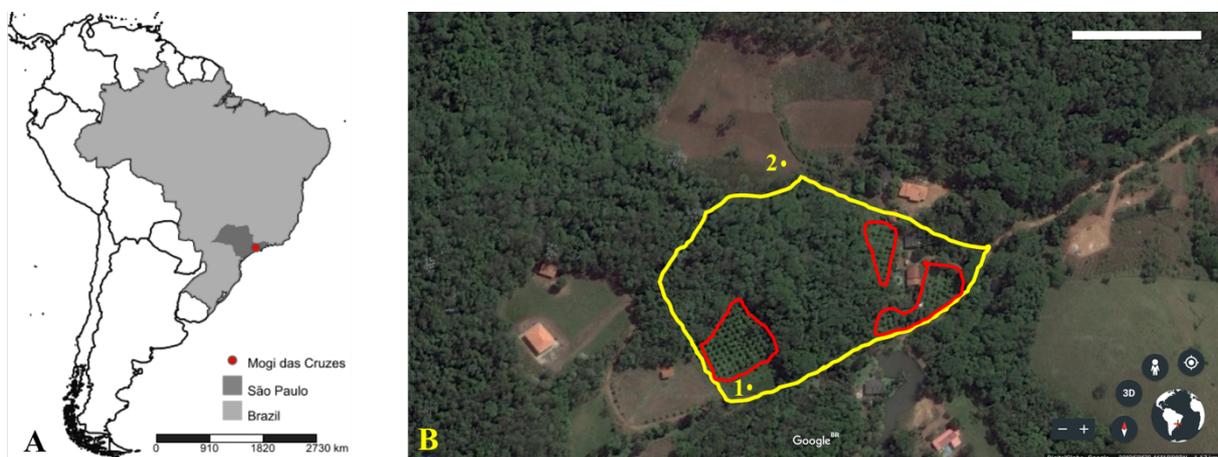
Nocturnal bees can be easily found foraging in a Neotropical plant during the morning twilight, which suits very well the goal of studying the possible light-dependent activity in nocturnal and crepuscular bees. *Campomanesia phaea* (O. Berg) Landrum 1984 is a Myrtaceae tree endemic to the Atlantic Forest in southeastern Brazil that reaches up to 5 m height, blooms between October and January, and produces an acidic and edible fruit commonly known as cambuci (Lorenzi 2002). The flowers open during the night releasing a sweet scent and are exclusively pollinated by four species of nocturnal and crepuscular bees, characterizing cambuci as one of the first records of a crepuscular melittophilous plant (Cordeiro *et al.* 2017). In the cambuci, it is common to hear several nocturnal bees visiting flowers during the twilight, making it a good system to our investigation.

Here we investigate how light intensity affects crepuscular bees foraging activity in cambuci flowers, controlling for other environmental factors that could interfere with this relation such as flower availability, temperature, humidity and wind speed. Our hypotheses are: 1) light intensity is the main environmental factor controlling crepuscular bees foraging activity; 2) crepuscular bees are more active in the intermediate light intensities of the twilight period. If we corroborate these hypotheses, we also expect that foraging activity starts earlier in brighter nights, such as moonlit nights. Our findings demonstrate how cambuci-crepuscular bees' interaction is strongly dependent on a single environmental factor. We discuss how this dependence can be explained by ecological requirements and evolutionary processes.

## Material & Methods

### Study area

Field work was conducted in a private farmland (Sítio Cambuci Nativa, 23°25'S, 46°10'W, 670 m above sea level) in the municipality of Mogi das Cruzes, São Paulo State, southeastern Brazil (Fig. 1a). The 15-ha farmland is located within the Atlantic Forest domain (Fig. 1b) and includes cambuci tree orchards (6 ha) and secondary forest fragments (9 ha). The orchards contain 237 cambuci trees divided between 15 juvenile trees lower than 1 m height and 222 young adult trees that can reach up to 5 m height. In the first half of the flowering season of 2017 (October-November), 215 adult trees produced flowers, representing more than 90% of the orchard. According to an updated Köppen-Geiger climate classification, the regional climate is type Cfa, namely characterized as humid subtropical, without a dry season, with a hot summer and average annual rainfall over 1400 mm (Peel *et al.* 2007).

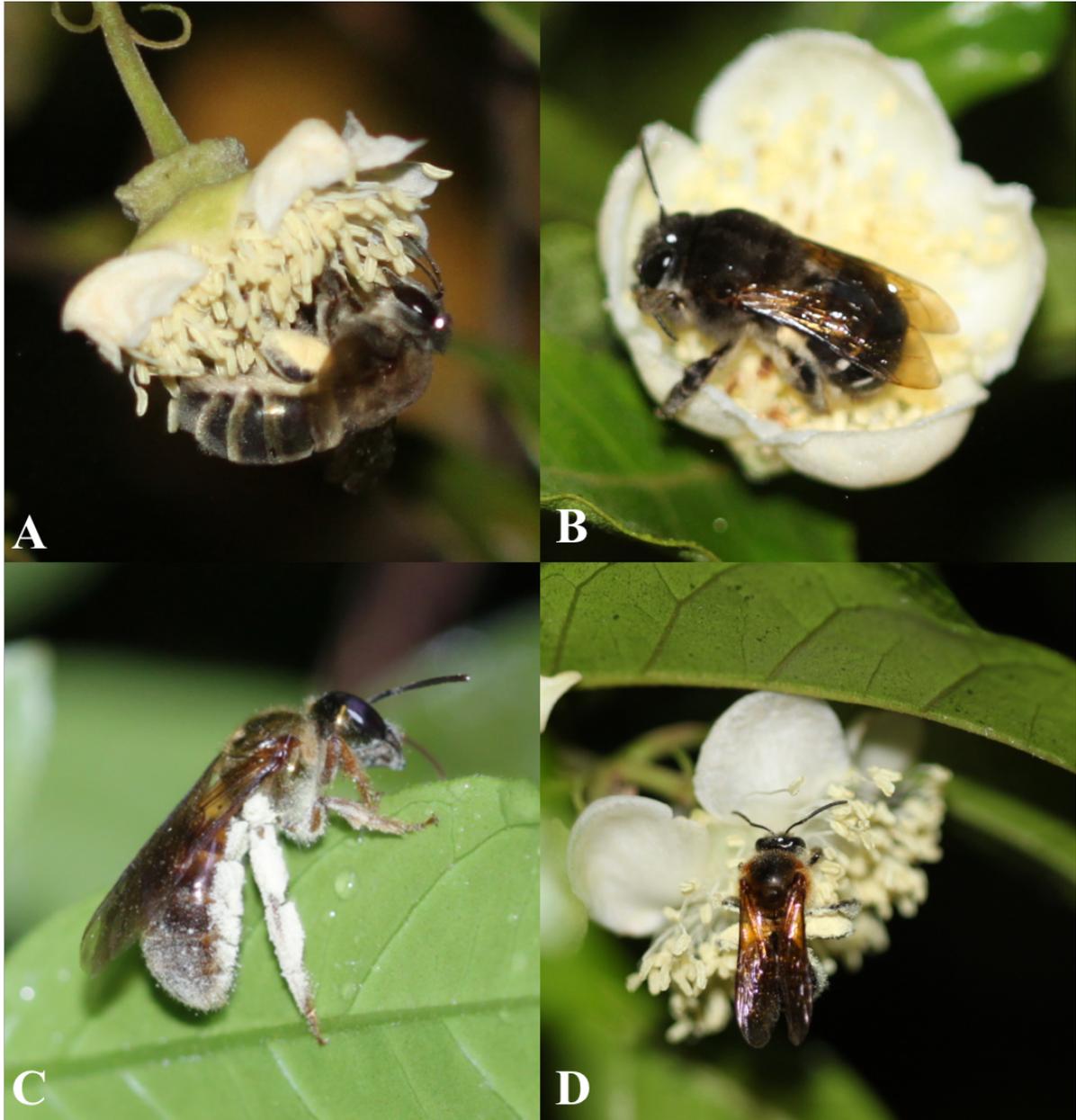


**Figure 1.** Study area location. A) South America map indicating Mogi das Cruzes city, São Paulo State, Brazil. B) Satellite picture from cambuci orchard study site (Source: Google Earth®). Yellow line delimits cambuci farmland and red lines delimit cambuci orchards surrounded by secondary Atlantic Forest fragments. Dots show location of 1) photometer (in an open area next to the orchard) and 2) meteorological station (in an open area 70-100 m away from orchards). White bar in the upper right corner equals 100 meters.

## Study system

Cambuci tree has a steady-state flowering strategy, producing just a few new flowers per day. The flowers are bisexual, three centimeters in diameter, plate type, with white petals and up to 500 stamens (Cordeiro *et al.* 2017). Floral anthesis lasts only one day and occurs during the true night, *i.e.*, before the beginning of the astronomical twilight (when sun elevation is less than  $-18^\circ$  relative to the horizon), between 4:00 and 4:30 a.m. (Brazilian Summer Time, BST). Pollen is the only reward offered, and collected by a large diversity of floral visitors, such as wasps, flies and bees, including nocturnal and crepuscular bees (Cordeiro *et al.* 2017). These insects visit cambuci flowers in different periods along anthesis; nocturnal bees initiate visits soon after floral anthesis and leave around sunrise, being followed by diurnal floral visitors until noon, when most of the pollen has already been depleted. According to visit exclusion tests, nocturnal bees are the only effective pollinators, characterizing cambuci pollination system as the first one with strong dependence on crepuscular and nocturnal bees (Cordeiro *et al.* 2017).

The study area has populations of at least four nocturnal and crepuscular bee species (Fig. 2) that visit cambuci flowers only at the morning twilight (before sunrise), but not at the evening twilight (after sunset): *Megalopta sodalis* Vachal 1904 and *Megommation insigne* Smith 1853 (Halictidae) and *Ptiloglossa latecalcarata* Moure 1945 and *Zikanapis seabrai* Moure 1953 (Colletidae). The former nests in wood sticks (Santos *et al.* 2010), while the other three nest in the soil (Michener and Lange 1958, Sarzetti *et al.* 2013). Both Colletidae species are crepuscular and solitary, while Halictidae species present some level of sociality and can be truly nocturnal – at least some species in *Megalopta* (Wcislo and Tierney 2009, Santos *et al.* 2010).



**Figure 2.** Crepuscular and nocturnal bee species that visit flowers of cambuci (*Campomanesia phaea*, Myrtaceae) in Southeastern Brazil. A) *Ptiloglossa latealcarata* (Colletidae), B) *Ptiloglossa* sp. (Colletidae), C) *Megalopta sodalis* (Halictidae), D) *Megommation insigne* (Halictidae).

### **Data collection**

#### *Flower visitation rate*

We counted flower visits per minute to measure bee foraging activity during the whole twilight of the morning. Also called dawn in its final part, this period begins when sun is 18°

below the horizon and starts to contribute to light levels in the atmosphere (ending the true night) and finishes when sun rises (Rozenberg 1966). We also recorded flower visits per minute in the beginning of the day until 30 minutes after sunrise (when sun elevation is around 6° above horizon and light intensity reaches a stable value), since crepuscular bees can extend their activity some minutes after sunrise in some days. We sampled 30 twilights during the first half of the cambuci flowering season, from mid-October to late November 2017. As twilight duration increases as solstice is approaching (Meinel and Meinel 1991), total observation time per twilight ranged from 108 to 115 minutes (mean  $\pm$  SD: 112  $\pm$  2; n=30). We did not sample rainy nights/twilights/mornings, since rain limits bee foraging activity and imposes technical limitations to measure light intensity levels.

During the true night, before anthesis (4:30 a.m., Brazilian Summer Time, BST) and before twilight, two or three observers randomly selected one tree each, totaling two or three flowering cambuci trees. The random choice of observed tree was repeated each night. When the sorted tree was not flowering, we moved in the planting line from east to west until a flowering individual was found. Each observer selected a fixed number of flowers to watch in each twilight (average of 12 observed flowers per tree, varying from 4 to 37, according to number of flowers in a suitable field of view) and stayed close to the tree waiting for the bees with a head lantern off. When one bee was heard, we turned lanterns on for some seconds to be able to correctly identify the bee species. We recorded number of visits for different bee species in each single minute. We calculated a flower visitation rate (number of visits per observed flower per minute) as our response variable, discriminated per tree and per night. We also counted visits from diurnal bee species since they started to arrive when crepuscular bees were still active.

### *Environmental predictors*

Light intensity was measured as luminance ( $\text{cd/m}^2$ ) of an 18% grey card by a very sensitive (measurement range:  $0.00001 - 19.90 \text{ cd/m}^2$ ) Photometer ERP-105 (Hagner, Sweden) coupled to a voltage data logger OM-CP-VOLT101A (Omega, Brazil), with sample rate of 5 seconds. Average light intensity per minute was recorded from the data logger and light data were measured for the entire observation period. The photometer was placed in an open area next to the orchard to avoid interference from observers' lanterns (Fig. 1b).

Other abiotic factors that could potentially affect bee activity were also measured to both control their effects and better discriminate the effect due to light intensity only. Air temperature and air relative humidity were measured per night with one thermohygrometer HOBO U23-001 (Onset, USA) installed at flower level in one of the observed cambuci trees. We also measured these climate variables with a meteorological station H21-USB (Onset, USA) installed in an open area 70-100 m away from the orchards (Fig. 1b). We measured maximum wind speed for the entire observation period with a portable cup anemometer PCE-A420 (PCE Instruments, Germany) installed in a platform close to the photometer. All devices were equipped with data loggers, and visitation data could be compared with environmental factors minute by minute. We also estimated cloud cover in the sky in each twilight; twenty nights had completely overcast skies while ten were clean and starlit nights. Weather conditions were relatively constant inside each observed twilight.

Flower availability was estimated as flower abundance by two ways: 1) as the total number of recently open flowers in 20 randomly selected adult cambuci trees each night (orchard flower abundance); and 2) as the total number of recently open flowers in the selected trees (tree flower abundance). We randomly selected 20 different individuals to count flowers after the end of the observation period and repeated the selection each night to detect daily floral

resources variation. We counted all recently open flowers in a tree following tree branches from bottom to top.

## Data analyses

To evaluate how light intensity affects crepuscular bees activity when controlling for other environmental factors, we modelled visit count data using generalized linear mixed models (GLMMs) with a Poisson distribution, assuming number of observed flowers per tree as an offset and using Laplace approximation to estimate parameters. All models included environmental factors (light intensity, temperature, air relative humidity, maximum wind speed and flower abundance) as predictor variables (fixed effects) and tree and night as possible random effects to account for nested sampling units.

Prior to all modelling, we standardized all explanatory variables and excluded operational variables that were highly correlated ( $\rho > 0.5$ ). Thus, we kept only temperature and humidity variables from the meteorological station and only orchard flower abundance estimative, excluding temperature and humidity data from thermohygrometer and tree flower abundance. All analyses were implemented in the R environment (R Core Team 2017) using the following extra packages: *bbmle* (Bolker and R Core Team 2017), *DHARMA* (Hartig 2017), *dplyr* (Wickham *et al.* 2017), *ggplot2* (Wickham 2009), *lme4* (Bates *et al.* 2015), *oce* (Kelley and Richards 2017), *tidyr* (Wickham and Henry 2018).

We followed model selection protocol from Zuur *et al.* (2009), starting with a full model, with all explanatory variables, to determine the best random-effect structure. We selected models using Akaike Information Criteria (AIC). We then kept the random-effect structure from the best model and constructed the following models to determine the best fixed-effect structure: 1) all environmental variables, except light; 2) all environmental variables, including light; 3) all environmental variables, including light and a quadratic term for light; 4) null model. We included a quadratic term for light intensity to model a scenario where

intermediate light intensities produce larger visitation rates. Then, we selected models using AIC again to detect what was the best relation between light intensity and crepuscular bees activity. We calculated 95% confidence intervals for each predictor variable in the best model to evaluate how strong the effect of light intensity was compared with other environmental factors. We expected the best model included light intensity and its quadratic term, indicating that intermediate light intensities are associated to higher activity during twilight. We also expected the light intensity parameters to be larger than other environmental factors.

We validated the best model using scalar (quantile) residuals created by a simulation-based approach implemented in the package DHARMA (Hartig 2017). We checked scaled residuals uniformity and plotted residuals against explanatory variables. We also checked zero-inflation. Validation occurred immediately after model selection and before results interpretation.

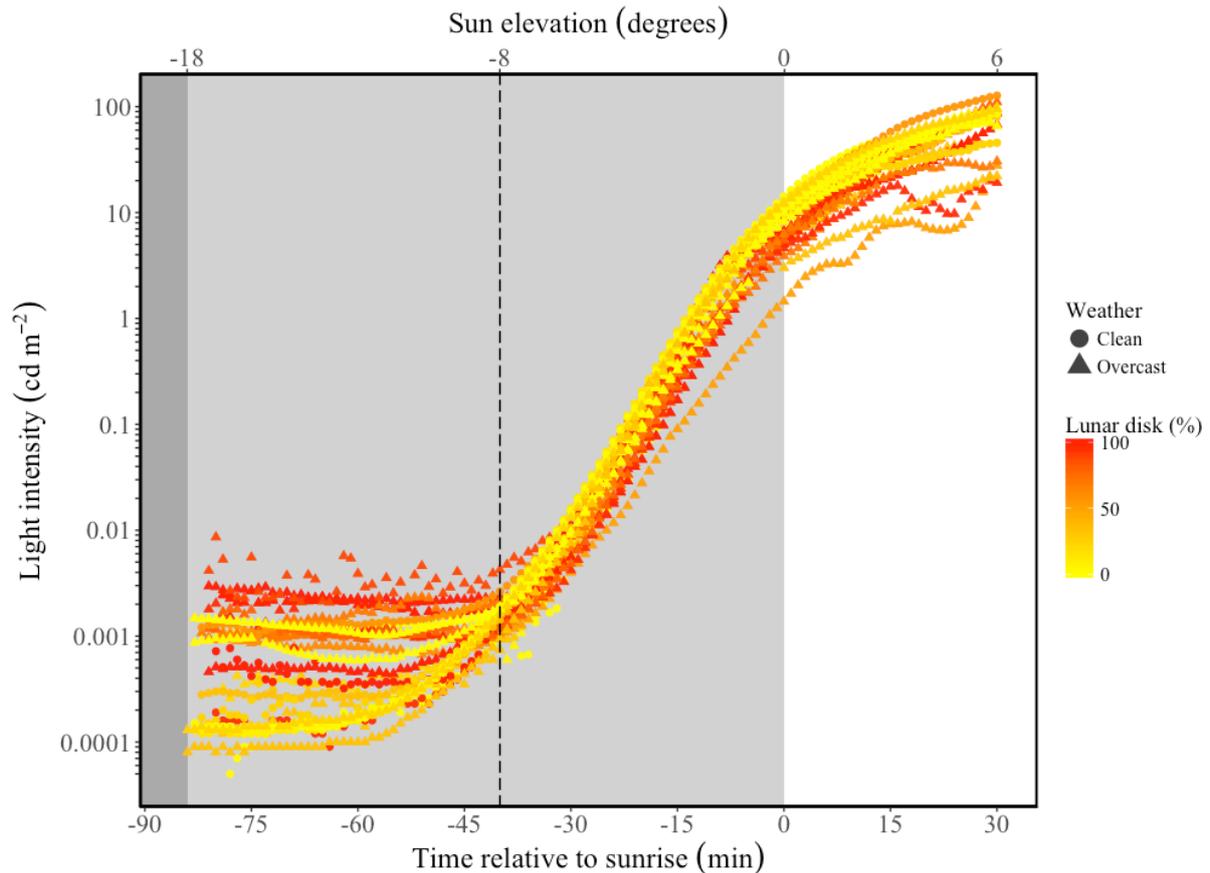
## **Results**

We observed flowers for 7614 minutes (or 127 hours) along 30 twilights. The beginning of the observation period varied from 5:12 a.m. (Brazilian Summer Time, BST) in the first twilight (October 17) to 4:45 a.m. (BST) in the last twilight (November 24), while the end of the observation period varied from 6:59 a.m. (BST) in the first twilight to 6:39 a.m. (BST) in the last twilight. This disparity reflects the variation in day length along season, which caused an increase of 7 minutes in the total observation period from the first (108 min) to the last (115 min) twilight.

## Environmental predictors

Light intensity levels varied in a constant shape during each twilight, following a sigmoid curve (Fig. 3). These levels are mainly controlled by sun elevation relative to the horizon. However, when sun elevation is lower than  $-8^\circ$ , *i.e.*, during the first half of the twilight, the contribution of sunlight to natural illumination is still low and, additionally, moon phase and/or weather conditions can significantly affect light intensity. Thus, we found variations of approximately two orders of magnitude in the light levels during the first half of the twilight among sampled twilights, *i.e.*, until approximately 40 minutes before sunrise (or sun elevation lower than  $-8^\circ$ ). This variation can be explained by a combination of moon phase and weather conditions, since a full moon can increase light levels during the night and the beginning of the twilight in almost two orders of magnitude compared with a starlit night, while cloud cover can decrease light levels in one order of magnitude (Fig. 3). After this initial variation, light levels become more similar among twilights because sun elevation is high enough to control and respond for almost all natural illumination.

Other environmental variables did not vary significantly during twilight period but presented different average values among twilights. Average temperature ranged from  $7.63^\circ\text{C}$  in the coldest twilight to  $18.75^\circ\text{C}$  in the warmest twilight (Fig. S1 – Appendix). All twilights were very humid and not windy, with average air relative humidity shifting from 89 to 100% (Fig. S2) and maximum wind speed varying from 0 to 10.3 km/h (Fig. S3). Flower abundance in the orchard increased along nights as the flowering season advanced, from 109 to 640 flowers (Fig. S4). Therefore, light intensity, temperature and flower abundance varied considerably among twilights, while air relative humidity and wind speed were more constant.



**Figure 3.** Light intensity measured as downwelling luminance ( $\text{cd}/\text{m}^2$ ) as a function of time relative to sunrise (bottom axis) and sun elevation relative to horizon (top axis) and discriminated by percentage of the illuminated lunar disk (full moon = 100%, new moon = 0%) and weather condition (clean sky or overcast sky). Each sigmoid curve represents a sampled twilight. Backgrounds colors represent night (dark grey), twilight (light grey) and daytime (white). The vertical dashed line marks the approximate half of the twilight. Sunrise time (0 in the axis-x) varied between 6:29 a.m. (Brazilian Summer Time, BST) in the first sampled twilight (Oct 17) to 6:09 a.m. (BST) in the last twilight (Nov 24).

### Visitation rate

Crepuscular bees made  $2.13 \cdot 10^{-2} \pm 6.46 \cdot 10^{-2}$  (mean  $\pm$  SD) visits per flower per minute (for all twilights) distributed in 33 cambuci trees. We identified five species of crepuscular bees from two families (Table 1). *Ptiloglossa latecalcarata* (Colletidae) was the most abundant species, with  $1.90 \cdot 10^{-2} \pm 6.12 \cdot 10^{-2}$  (mean  $\pm$  SD) visits per flower per minute – approximately

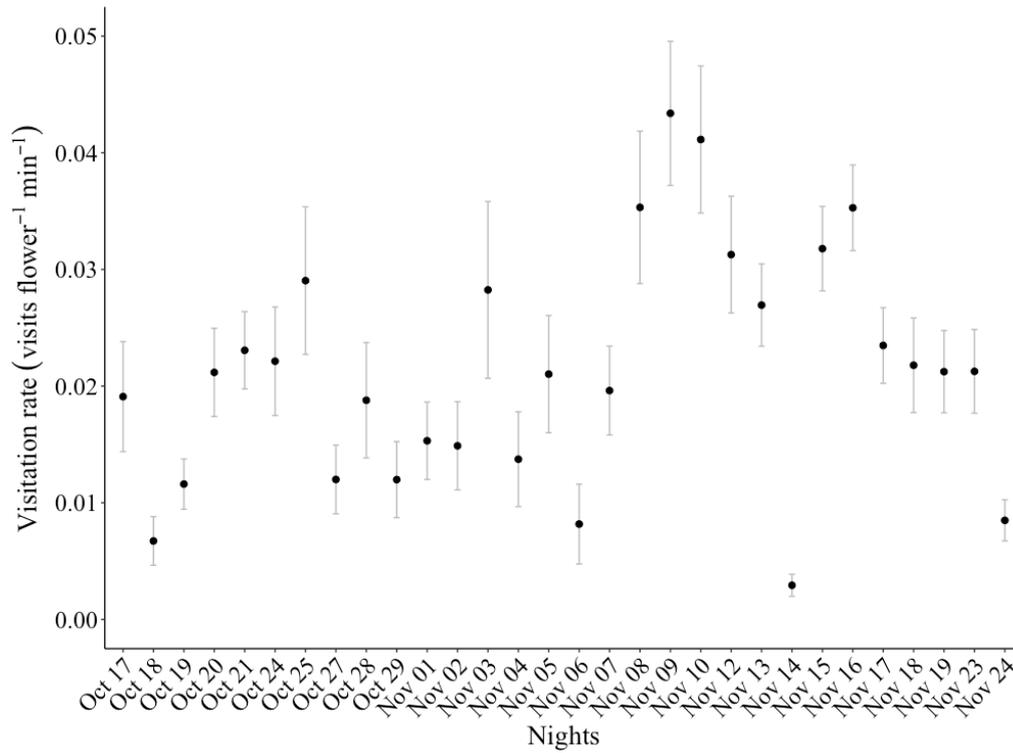
89% of the total, reflecting also the faster flower visitation behavior of this species of around two seconds. All the other species were considerably rarer.

**Table 1.** Visitation rate (mean  $\pm$  SD) of five crepuscular bee species foraging in a commercial cambuci (*Campomanesia phaea* – Myrtaceae) orchard surrounded by secondary Atlantic Forest fragments in Mogi das Cruzes, São Paulo State, Brazil.

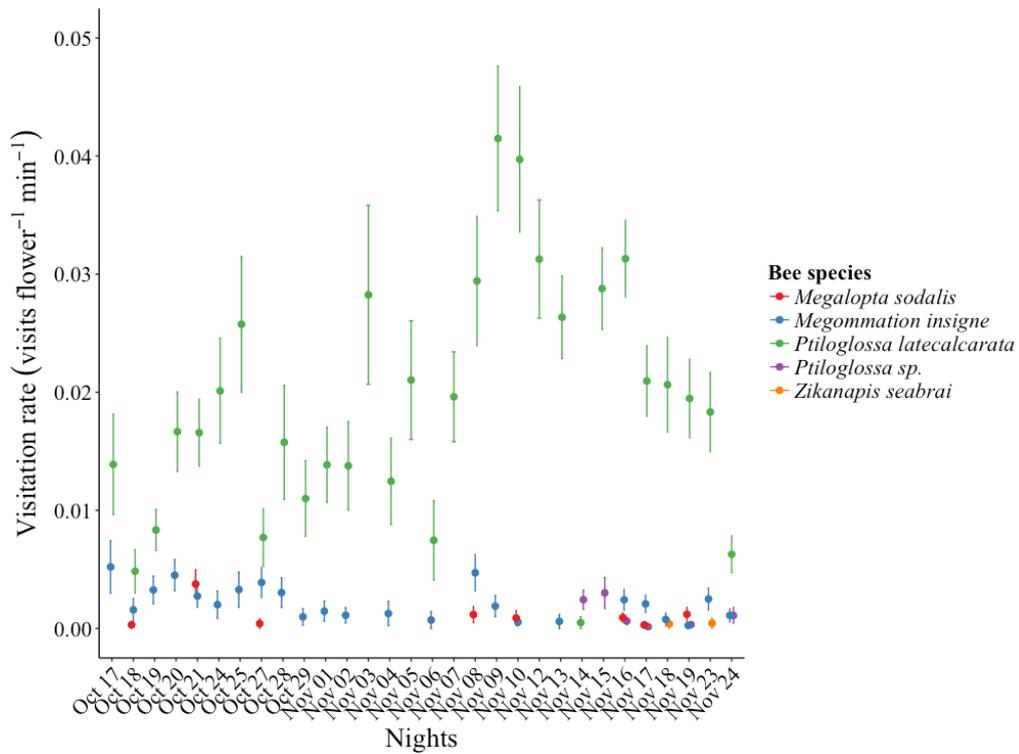
Bee family	Bee species	Visitation rate ( $10^{-2}$ visits/flower/min)
Colletidae	<i>Ptiloglossa latecalcarata</i>	1.90 $\pm$ 6.12
	<i>Ptiloglossa</i> sp.	0.03 $\pm$ 0.66
	<i>Zikanapis seabrai</i>	0.002 $\pm$ 0.15
Halictidae	<i>Megalopta sodalis</i>	0.03 $\pm$ 0.51
	<i>Megommation insigne</i>	0.17 $\pm$ 1.39
<b>Total</b>		2.13 $\pm$ 6.46

Visitation rate, in average, did not increase as cambuci flowering season advanced ( $p = 0.17$ ,  $r^2 = 0.03$ ; Fig. 4), mainly due to *P. latecalcarata* frequent activity (Fig. 5). The other bee species showed lower frequencies, with two species (*Ptiloglossa* sp. and *Z. seabrai*) appearing only in the last twilights. In the most active night (November 9), bees made almost 15 times more visits per flower per minute in average than in the less active one (November 14) (Fig. 4).

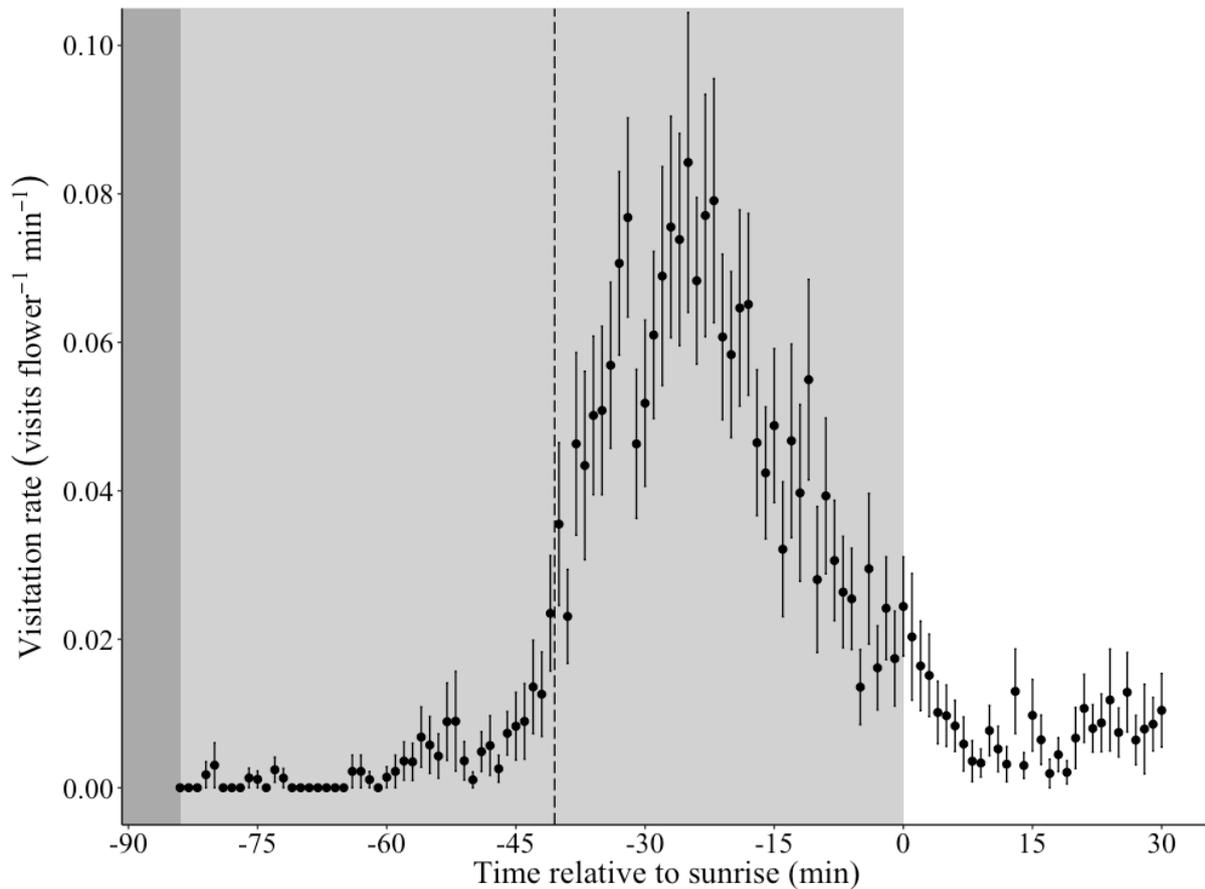
During twilight, visitation rate increased and reached a peak around 30 minutes before sunrise, then decreased (Fig. 6; Fig. S5). Visitation rate started to increase significantly around the half of the twilight, in the same time that light intensity started to increase too (compare data from Fig. 3 and Fig. 6 close to the dashed line). Most crepuscular bee species stopped foraging in cambuci around sunrise, but *P. latecalcarata* extended its activity significantly until 30 minutes after sunrise in some days (Fig. S6; Fig. S7). Diurnal bees, mostly *Apis mellifera* (Apidae) but also some stingless bees (Meliponini) species, started to visit cambuci flowers around 30 minutes before sunrise (Fig. S8; Fig. S9).



**Figure 4.** Visitation rate (visits/flower/min) from crepuscular bees in cambuci flowers along sampled nights. Dots are average values and bars are standard errors of the mean.



**Figure 5.** Visitation rate (visits/flower/min) from five crepuscular bees species in cambuci flowers along sampled nights. Dots are average values and bars are standard errors of the mean.



**Figure 6.** Visitation rate (visits/flower/min) from crepuscular bees in cambuci flowers during twilight period and the beginning of the morning. Dots are average values and bars are standard errors of the mean. Background colors represent true night (dark grey), twilight (light grey) and daytime (white). The vertical dashed line marks the approximate half of the twilight. Sunrise time (0 in the axis-x) varied between 6:29 a.m. (Brazilian Summer Time, BST) in the first sampled twilight (Oct 17) to 6:09 a.m. (BST) in the last twilight (Nov 24).

### **Light intensity affects visitation rate by crepuscular bees in cambuci flowers**

The only environmental factor explaining cambuci flower visitation rate by crepuscular bees was light intensity. The model with light effect and a quadratic term for light was the best model (Table 2), with the following equation for standardized variables:

$$\begin{aligned} \text{Visitation rate} = & -2.94 + 0.44*(\text{light intensity}) - 1.60*(\text{light intensity})^2 \\ & - 0.05*(\text{temperature}) + 0.06*(\text{humidity}) - 0.07*(\text{wind}) + 0.08*(\text{flowers}) \end{aligned}$$

**Table 2.** Model selection for factors affecting crepuscular bees foraging activity, using generalized linear mixed models (GLMMs) with Poisson distribution. Response variable was number of visits per minute, pondered by number of observed flowers as model offset. The best model includes all environmental variables (light intensity, temperature, air relative humidity, maximum wind speed, flower abundance) and a quadratic term for light intensity. AIC = Akaike information criterion; DF = degrees of freedom.

<b>Models</b>	<b>logLikelihood</b>	<b>AIC</b>	<b>ΔAIC</b>	<b>DF</b>	<b>Weight</b>
Light + light <sup>2</sup> + all other variables	-3645.5	7309.1	0.0	9	1
Light + all other variables	-4379.6	8775.1	1466.1	8	< 0.001
All variables, except light	-4381.0	8775.9	1466.9	7	< 0.001
Null	-4670.4	9342.7	2033.6	1	< 0.001

**Table 3.** Summary statistics of the best generalized linear mixed model that includes all variables and a quadratic term for light intensity. SE = standard error.

<b>Variable</b>	<b>Estimate</b>	<b>SE</b>	<b>z value</b>	<b>p value</b>
Intercept	-2.93789	0.17685	-16.61	< 2 · 10 <sup>-16</sup>
Light	0.44242	0.03781	11.70	< 2 · 10 <sup>-16</sup>
Light <sup>2</sup>	-1.60231	0.04798	-33.39	< 2 · 10 <sup>-16</sup>
Temperature	-0.05550	0.14160	-0.39	0.695
Humidity	0.06368	0.11995	0.53	0.596
Wind speed	-0.06928	0.11362	-0.61	0.542
Flower abundance	0.07817	0.12074	0.65	0.517

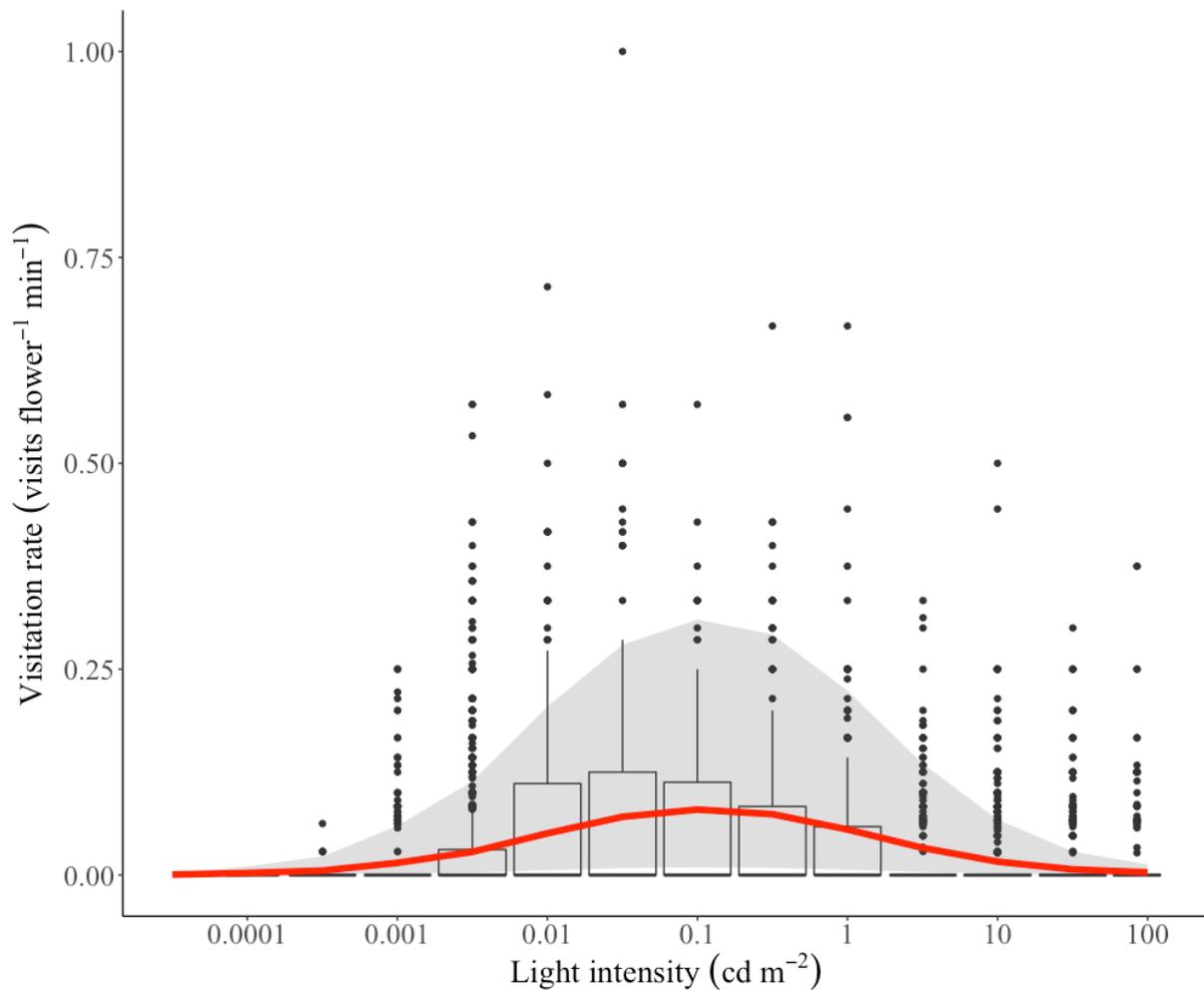
Model coefficients indicated that light intensity has a strong effect, while all the other environmental variables presented weak effects (Table 3). Light intensity had an effect at least 5.5 (0.44/0.08) times larger than the second most important variable (flower abundance). The 95% confidence intervals for estimated parameters showed that effects from temperature, air humidity, maximum wind speed and flower abundance are so low that they are not different from zero (Table 4). Therefore, only light intensity had a significant effect on visitation rate.

**Table 4.** The 95% confidence intervals for the estimates of each predictor variable (fixed effects) of the best generalized linear mixed model. Intervals for tree and night (random effects) are for their standard deviations, which were estimated as 0.71 and 0.60, respectively.

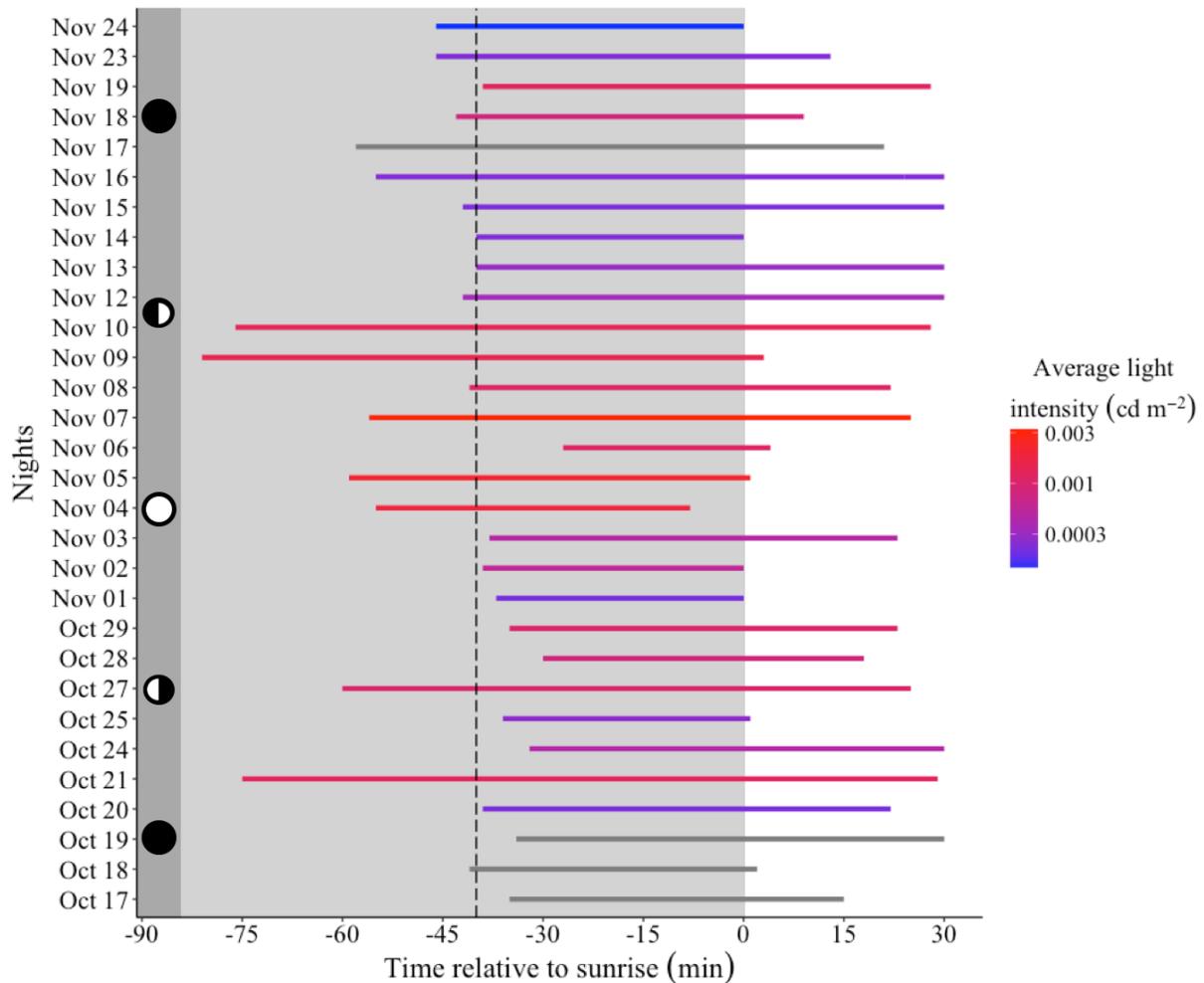
<b>Variables</b>	<b>2.5 %</b>	<b>97.5 %</b>
Intercept	-3.3028075	-2.5894535
Light	0.3680751	0.5173758
Light <sup>2</sup>	-1.6979153	-1.5085645
Temperature	-0.3348396	0.2404028
Humidity	-0.1558794	0.3245145
Wind speed	-0.3038869	0.1590065
Flower abundance	-0.1718659	0.3196936
Tree	0.5118254	1.0106558
Night	0.4397358	0.8587097

We also found that visitation rate peaks at intermediate light intensity levels (Fig. 7), as we expected. According to the best model, at a medium light intensity of 0.01 cd/m<sup>2</sup> bees would make 32 times more visits per flower per minute than the at the lowest level of 0.0001 cd/m<sup>2</sup> and 24 times more visits per flower per minute than at the highest level of 100 cd/m<sup>2</sup>. Besides, we could determine the minimum light level (of 0.00024 cd/m<sup>2</sup>) when crepuscular bees started to forage in cambuci flowers.

We could also deduce from the selected model that brighter nights, *i.e.*, the ones with higher light intensities levels during the first half of the twilight (Fig. 3), can incite earlier visits. Initial light intensity levels found in brighter nights – around 0.003 cd/m<sup>2</sup> – are responsible for increased crepuscular bees activity when compared to initial light intensity levels in darker nights – around 0.0003 cd/m<sup>2</sup> (see the response of visitation rate to these light intensity levels in the red curve in Fig. 7). Indeed, nights with earlier crepuscular bees activity seem to be brighter nights (Fig. 8), which were defined by average light intensity only during the first half of the twilight, when light levels varied significantly among twilights (Fig. 3).



**Figure 7.** Distribution of visitation rate (visits/flower/min) from crepuscular bees in cambuci flowers during twilight as a function of light intensity measured as downwelling luminance (cd/m<sup>2</sup>). Although axis-x is in log scale and data are aggregated in intervals of  $5 \log_{10}(\text{cd/m}^2)$ , axis-x labels are in linear scale for better understanding. Boxes delimit where 50% of the values for each light interval are concentrated, horizontal lines indicate the median (all of them are zero), and dots represent outliers. Red curve represents the best model that includes a quadratic term for the light effect. The 95% confidence interval is showed in light grey.



**Figure 8.** Crepuscular bees foraging activity ranges in cambuci flowers along twilights. Each horizontal line represents the time interval when bees were visiting cambuci in each twilight. Line colors indicate average light intensity – measured as downwelling luminance ( $\text{cd}/\text{m}^2$ ) – only from the first half of the twilight, which is when light levels varied between nights (see Fig. 3). The vertical dashed line marks the approximate half of the twilight. Grey lines are nights with missing light intensity data from the first half of the twilight. Background colors represent night (dark grey), twilight (light grey) and daytime (white). Sunrise time (0 in the axis-x) varied between 6:29 a.m. (Brazilian Summer Time, BST) in the first sampled twilight (October 17) to 6:09 a.m. (BST) in the last twilight (November 24). Circles on the left side represent moon phases (new moon = black circle; full moon = white circle).

## Discussion

The foraging activity of crepuscular bees in cambuci flowers during morning twilight was explained only by light intensity. Their maximum activity occurs during intermediate light intensity levels (around  $0.1 \text{ cd/m}^2$ ), which usually happen 30 minutes before sunrise. The onset of activity occurs after a minimum light intensity threshold (of  $0.00024 \text{ cd/m}^2$ ), which is the first record for crepuscular bees finding flowers in an agro-forest context. Besides, at twilights with higher initial light intensity levels, such as during clear moonlit nights, crepuscular bees can anticipate the beginning of their foraging time. This light-dependent foraging activity is likely related to a combination of factors such as visual challenges imposed by dim light levels and ecological demands to access resources before the massive visit of diurnal bees.

It makes sense that light intensity controls crepuscular bees activity because dim light environments represent an extreme situation for bees, which are equipped with apposition compound eyes that work better in brighter conditions (Warrant 2008, Warrant and Dacke 2011). Thus, we expected that a minimum light intensity threshold operates over the visual capabilities of crepuscular bees and, consequently, over their activity. This threshold does exist for some nocturnal and crepuscular species. Kelber *et al.* (2006) have found that the low light intensity of  $0.0001 \text{ cd/m}^2$  limits foraging activity for *Megalopta genalis* (Halictidae), a nocturnal bee from forests, and *Lasioglossum (Sphecodogastra) lusoria* (Halictidae), a crepuscular bee from deserts. However, their major findings focus on how much light bees need to find their nests when returning home, placing light effect over foraging in a nest-context for *M. genalis*. A bee could be able to find the nest but not find a flower at a low light level. Thus, it was still an open question how light intensity could affect foraging activity *per se* during foraging trips in flowers. Here, for the first time, we present a minimum light intensity value for bees finding flowers in an agro-forest context, where shadows from neighbor taller trees reduce light levels even more. Our findings agree with the previous reported threshold for a

forest context (Kelber *et al.* 2006), since our first visits were recorded after this same low light level measured for *M. genalis*. That indicates crepuscular bees foraging on cambuci could also have a similar threshold. Surprisingly, though, our registered minimum threshold – of 0.00024 cd/m<sup>2</sup> – is at least 10 times dimmer than the value registered by Kelber *et al.* (2006) for the desert crepuscular bee *L. lusoria* foraging in Onagraceae plants. This finding expands our previous understanding about the dimmest conditions where crepuscular bees can find flowers in general. Thus, our results demonstrate that the minimum light intensity threshold also operates in foraging activity on flowers and in an agro-forest context, even at dimmer levels than previously known for other environments.

Besides using vision, crepuscular bees also orient themselves to flowers using cambuci floral volatiles, mainly 1-octanol that is more emitted during the night, soon after anthesis (Cordeiro *et al.* 2017). Combining these previous results with our findings of a minimum light intensity threshold operating in flower search behavior, and therefore, indicating a vision component in this task, we can deduce that crepuscular bees should use a multisensorial input to find flowers. Diurnal bees combine visual and olfactory inputs to recognize and find flowers (Burger *et al.* 2010, Milet-Pinheiro *et al.* 2012), but now, for the first time, we have indications this can be the case for crepuscular bees too. Thus, it can be worthy to investigate next how crepuscular and nocturnal bees respond specifically to several visual cues in flowers and then try to answer directly how these bees integrate different sensory information (vision, olfaction, etc.) in their foraging behaviors.

Even though bees can use other sensory information to find flowers in the dark, such as floral volatiles (Cordeiro *et al.* 2017), they can still depend heavily on visual cues to learn the first flowers and to land on them (Orbán and Plowright 2014). Moreover, another bee behavior that is also visually guided, and therefore light-dependent, is the search for mates (Michener 2007). In species that rely on vision to primarily finding reproductive partners, male bees can

have larger eyes than females (Somanathan *et al.* 2017). We know at least for *P. latecalcarata* that males seem to have larger eyes than females (personal observation), indicating a possible visually dependent searching behavior. Therefore, all these visually guided behaviors support a strong bee light-dependent activity in general.

Our results also show that light intensity influences crepuscular bees activity in a non-linear relation, contrasting with what we know for diurnal bees (Michener 2007, Polatto *et al.* 2014). Many studies have demonstrated how light intensity affects the beginning of the foraging activity in diurnal bees (*e.g.*, Linsley 1958, Willis and Kevan 1995, Wcislo and Cane 1996, Figueiredo-Mecca *et al.* 2013). It is generally stated that light controls bees activity in a linear way (Polatto *et al.* 2014), and consequently, cloudy days should delay the onset of bees activity, which was confirmed for several species of solitary and social bees (Linsley 1958, Figueiredo-Mecca *et al.* 2013, Streinzer *et al.* 2016). However, this is not true for crepuscular bees. They still have a strong light-dependent activity, but intermediate light levels increase crepuscular bees activity while more light after a peak decreases their activity. Therefore, we propose a reformulation of this light-dependent activity model by adding the crepuscular bees case.

A reformulation of the light-dependent activity model asks for an explanation of why higher light intensities could actually decrease the activity of crepuscular bees. We propose two non-exclusive possibilities. First, crepuscular bees can have an endogenous clock that orients them about the exact time to start and to stop foraging, as it has already been demonstrated for other bee species (Bellusci and Marques 2001, Bloch *et al.* 2017). Using environmental cues such as light intensity to regulate a circadian rhythm (Enright 1970), bees could predict when is the best time to stop collecting more resources and start to invest in activities in the nest. Second, higher light levels trigger or coincide with the arrival of diurnal bees in cambuci flowers, possibly stimulating crepuscular bees to stop their foraging activity to avoid competition. Originally, nocturnal and crepuscular bees might have changed their preferred

foraging period from daytime to nighttime because nocturnal and crepuscular niches could offer less competition and an enemy-free space (Wcislo and Tierney 2009). Wcislo *et al.* (2004) tested this hypothesis for two Neotropical nocturnal bee species in Panama – *Megalopta genalis* and *M. ecuadoria* (Halictidae) – and they found nest parasitism rates for these species were four times lower when compared with the rates of closely related diurnal species. Besides, Smith *et al.* (2017) have found evidences that interference competition with diurnal bees ceases *Megalopta* foraging activity in the morning. We have also observed that diurnal bees, mainly *Apis mellifera*, arrive in large numbers soon before sunrise and occupy all the space in flowers (Figs. S8; S9), which could have deterred the activity of crepuscular bees. Therefore, our results support the idea that higher light intensities can be used as a proxy for the end of an activity regulated by circadian clock and/or an increased competition with diurnal bees, since higher light levels did not avoid crepuscular bees to continue seeing their surroundings (Eric Warrant, personal communication). But at light levels of 100 cd/m<sup>2</sup> (around 30 minutes after sunrise) bees do cease their activity, which can evolutionary be set to finish in the same time of the day and coincides with the arrival of many diurnal species, especially social bees, on cambuci flowers (Cordeiro *et al.* 2017; and Figs. S8; S9 here).

Another interesting aspect of our results is the fact that light intensity seems to be the only factor controlling the activity of crepuscular bees. Considering diurnal bees usually also respond to temperature and flower availability (Linsley 1958, 1978), then why does flower availability not affect crepuscular bees? As previously stated, nocturnal and crepuscular lifestyle brought some advantages to access resources earlier than potential competitors (Wcislo *et al.* 2004), so flower limitation due to competition should be lower, and therefore, not affect crepuscular bees activity as much as seen for diurnal bees. Besides, in our agroforest context, bees were foraging in a large food site with high density of cambuci trees in a small area and resource limitation should not be an impediment to their activity.

Additionally, why does temperature not affect crepuscular bees in cambuci? As far as we know, only four studies (Shelly *et al.* 1993, Gottlieb *et al.* 2005, Franco and Gimenes 2011, Oliveira *et al.* 2016) have evaluated in some way how temperature determine nocturnal bees activity. Two of them (Shelly *et al.* 1993, Oliveira *et al.* 2016) have found no effect of temperature, one has implicated that temperature affects positively bee activity over a range of 30°C (Gottlieb *et al.* 2005) and another has found that temperature has a moderate negative effect over activity (Franco and Gimenes 2011). Three of them have also suggested light intensity as a factor affecting bees activity (Shelly *et al.* 1993, Gottlieb *et al.* 2005, Franco and Gimenes 2011). Our study seems to be the first one to evaluate how several factors can simultaneously affect nocturnal bees activity in a fine scale (of one minute) and to determine the exact relation, in terms of magnitude and direction, between environmental variables and crepuscular bee foraging activity. Thus, in general, it seems light intensity affects the activity of crepuscular bees in several cases, but a temperature effect varies according to context. As temperature is relatively constant in tropical environments (Borges *et al.* 2016), light could be the only determinant factor to Neotropical nocturnal bees. In our case, although cambuci crepuscular bees experienced not so constant temperatures – varying from approximately 8 to 19 °C – bees were able to visit flowers even in the coldest night. This suggests that low temperature is not an obstacle here and that perhaps crepuscular bees could do thermoregulation in some way. Many desert bees have a bimodal activity pattern similar to crepuscular bees and are notably able to overcome the low temperature in the beginning of the morning and to avoid overheating in the hottest hours of the day, being classified as endothermic animals (Willmer and Stone 1997). For instance, the desert crepuscular bee *Ptiloglossa arizonensis* can fly very early in cold days and, once in the air, this bee can keep higher thoracic temperatures than the surroundings (Shelly *et al.* 1993). As our results are strongly biased to *Ptiloglossa*

*latecalcarata*, which is closely related to *P. arizonensis* and should have the same thermoregulatory capacities, temperature also seems to play a minor role here.

Finally, a major implication of our results is that as light intensity strongly influences the activity of crepuscular bees, any possible changes of light levels such as caused by local light pollution could interfere with bees activity, and consequently, with cambuci pollination. Since nocturnal bees are naturally attracted to artificial light, which supports traditional collection methods using light traps, significant light pollution such as strong local artificial sources (*e.g.*, farm's outdoor lightning) could confound bees and attract them to places far away from flowers. However, it would be interesting to investigate if low increases in light levels could actually increase their activity in the first half of the twilight by reducing their visual limitations and thus, contribute to increased cambuci pollination. A general increase in light levels caused by diffuse light pollution, such as coming from nearby towns, can also affect crepuscular bees activity in a more consistent way. For instance, Knop *et al.* (2017) found that artificial light pollution at night (ALAN) can reduce in 62% the visits of nocturnal pollinators to their host plants. Future studies should quantify how these different types of light pollution (local and diffuse) can disturb nocturnal and crepuscular bees activity.

## **Conclusion**

In conclusion, light intensity is the only environmental factor affecting bees foraging activity in cambuci flowers. Their activity during morning twilight peaks at intermediate light intensity levels. This implies that brighter nights, with higher light levels in the first half of the twilight, permit earlier foraging activity in cambuci flowers. These findings highlight for the first time what environmental factors are relevant to explain the interaction between crepuscular bees and their host plants. Our results also change the current opinion that bees light-dependent activity is always linear. In the case of nocturnal bees, we have found a nonlinear model that

could be explained in terms of the evolutionary advantages to forage earlier, associated with a possible endogenous clock marking the time to stop foraging and/or the competition of diurnal bees at higher light levels. Further studies need to test how light pollution can disturb this light-dependent activity and to focus on expanding our understanding about how bees, including diurnal species, rely on light levels to forage in other contexts.

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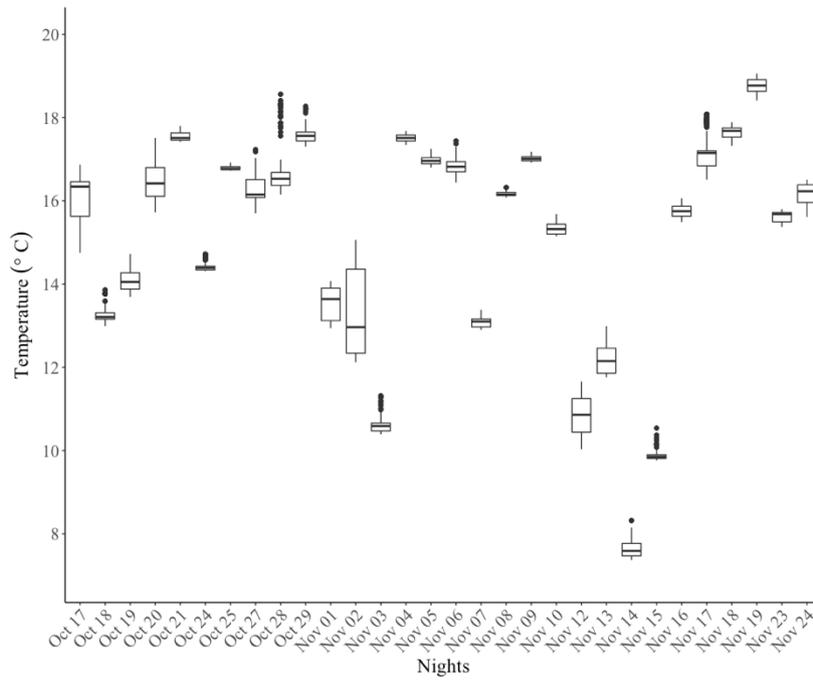
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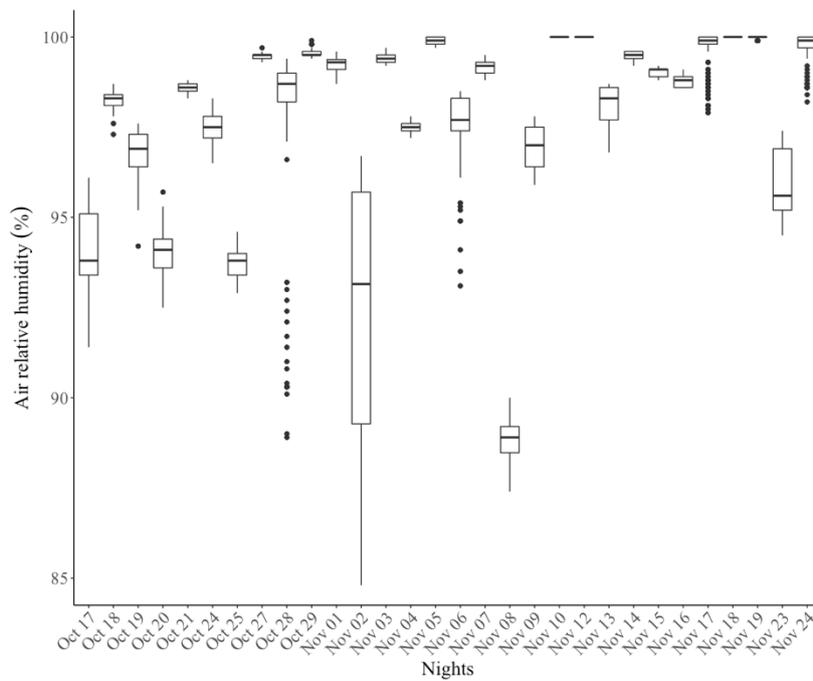
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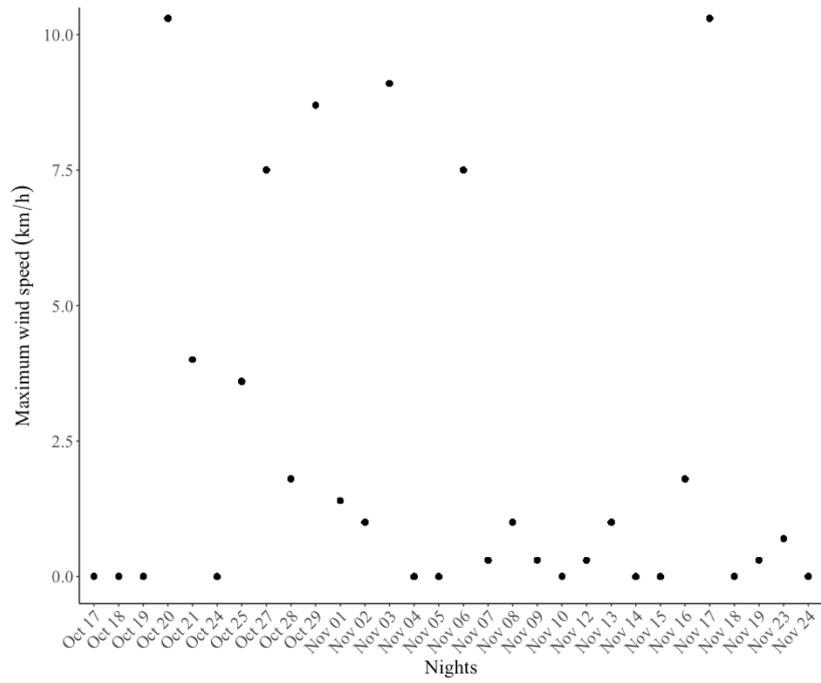
# Appendix



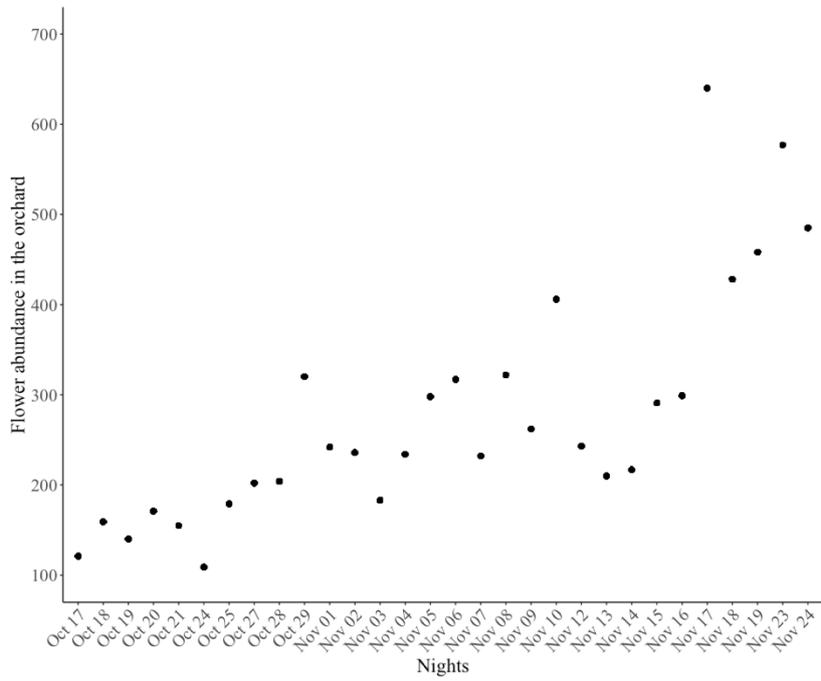
**Figure S1.** Temperature (°C) distribution along sampled twilights. Boxes delimit where 50% of the values are concentrated, horizontal lines indicate the median, and dots represent outliers.



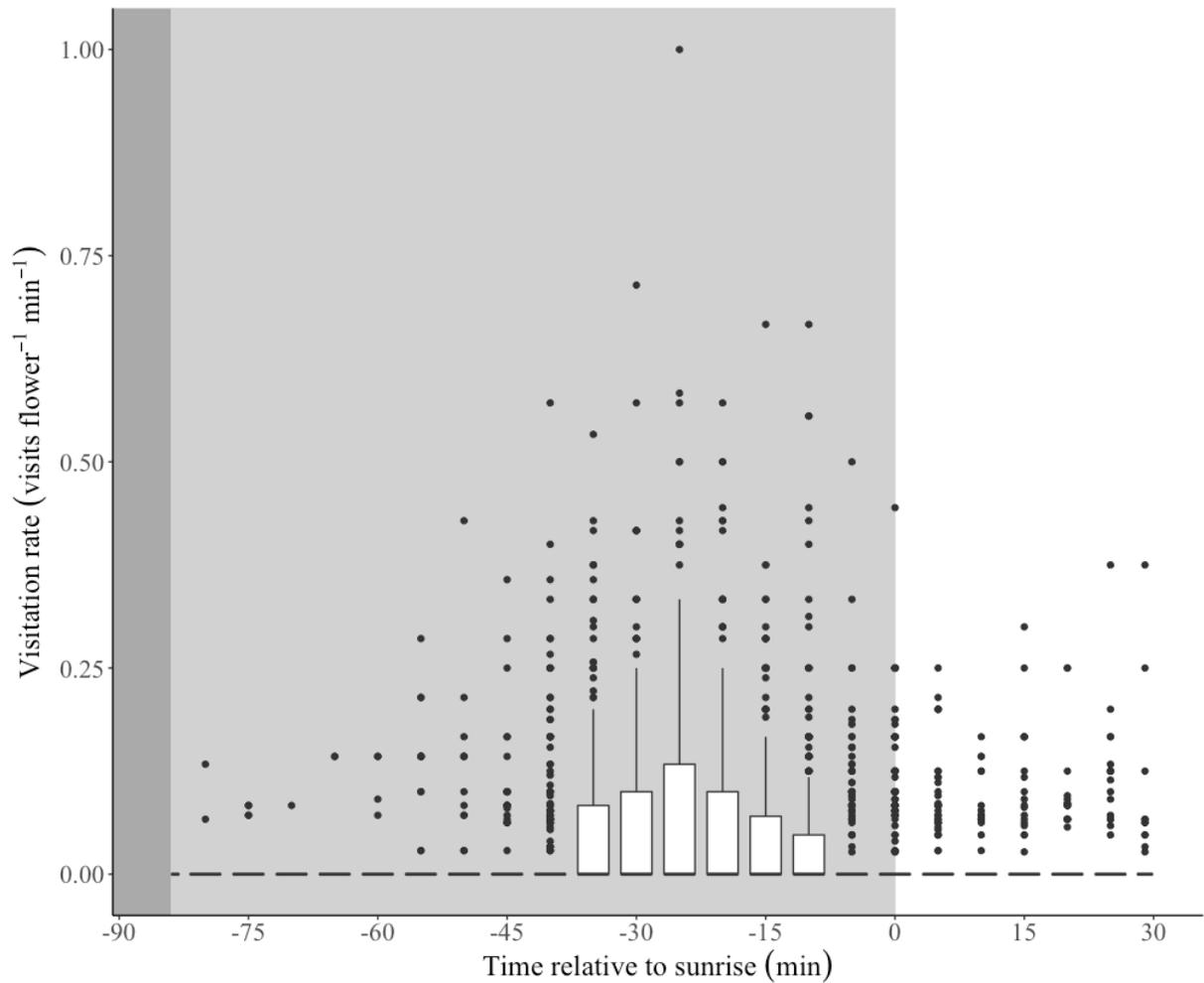
**Figure S2.** Air relative humidity (%) along sampled twilights. Boxes delimit where 50% of the values are concentrated, horizontal lines indicate the median, and dots represent outliers.



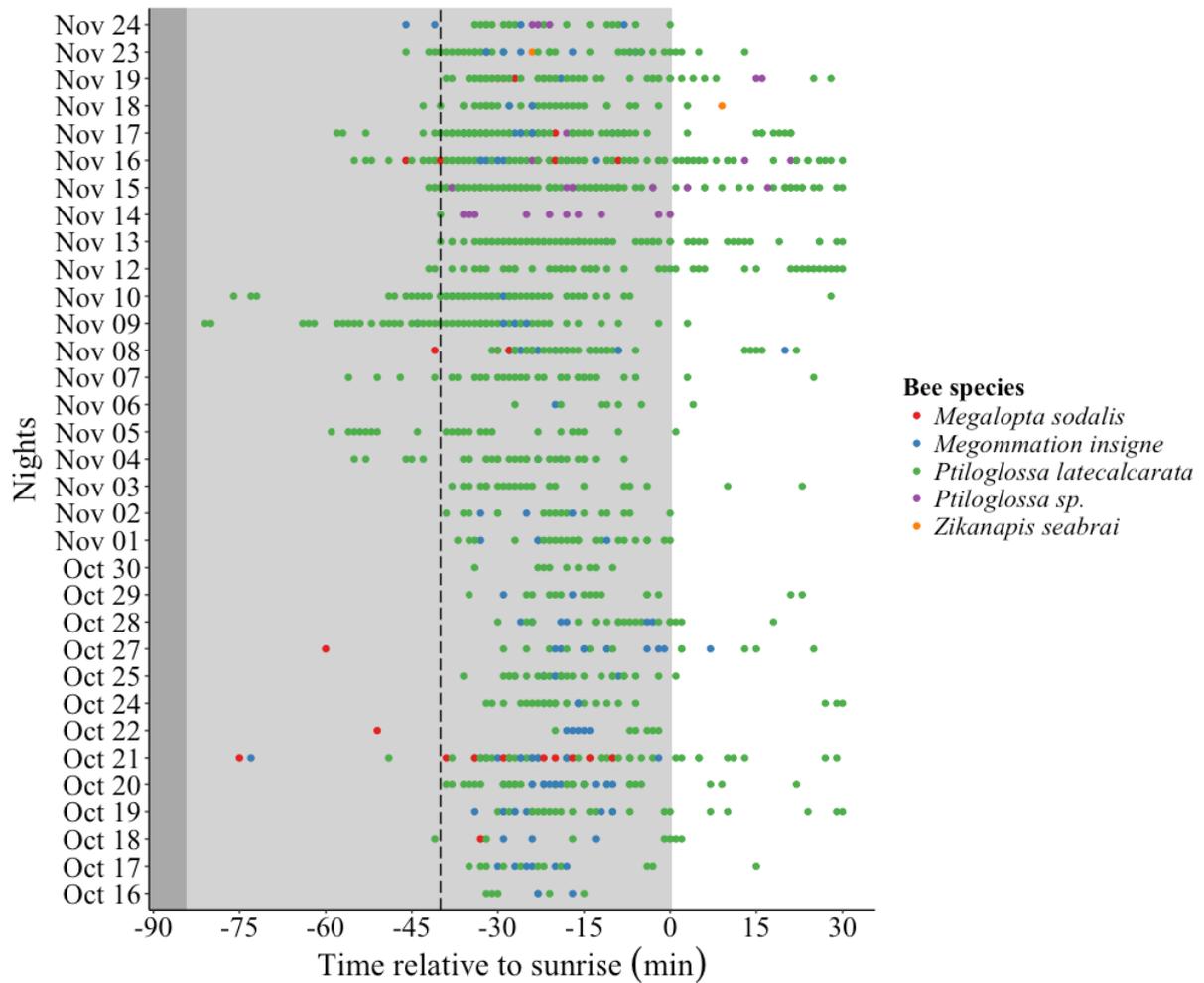
**Figure S3.** Maximum wind speed (km/h) along sampled twilights. Measurements were integrated along the entire observation period for each twilight, which varied between 108 and 115 minutes (twilight length).



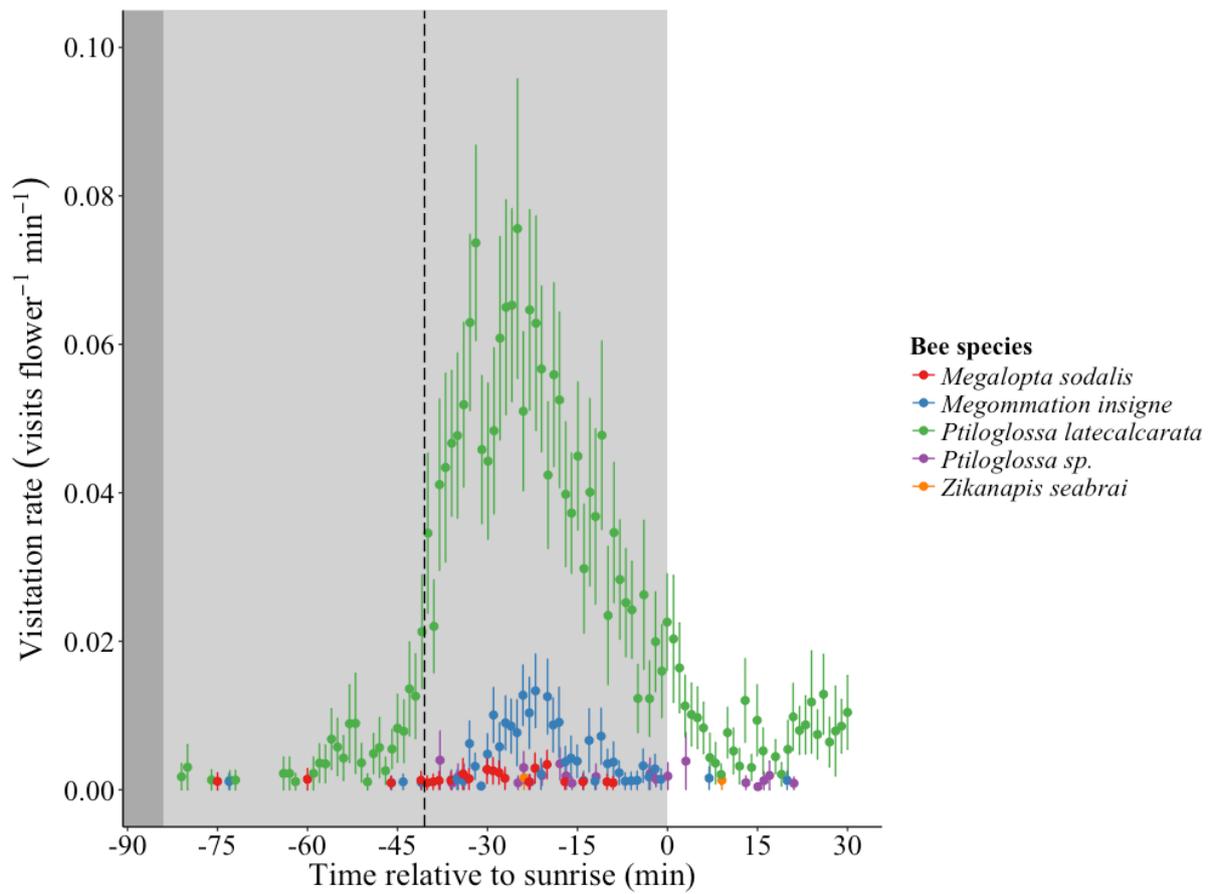
**Figure S4.** Flower abundance of the cambuci orchard along sampled nights. Estimative based on total number of open flowers from 20 randomly selected cambuci trees.



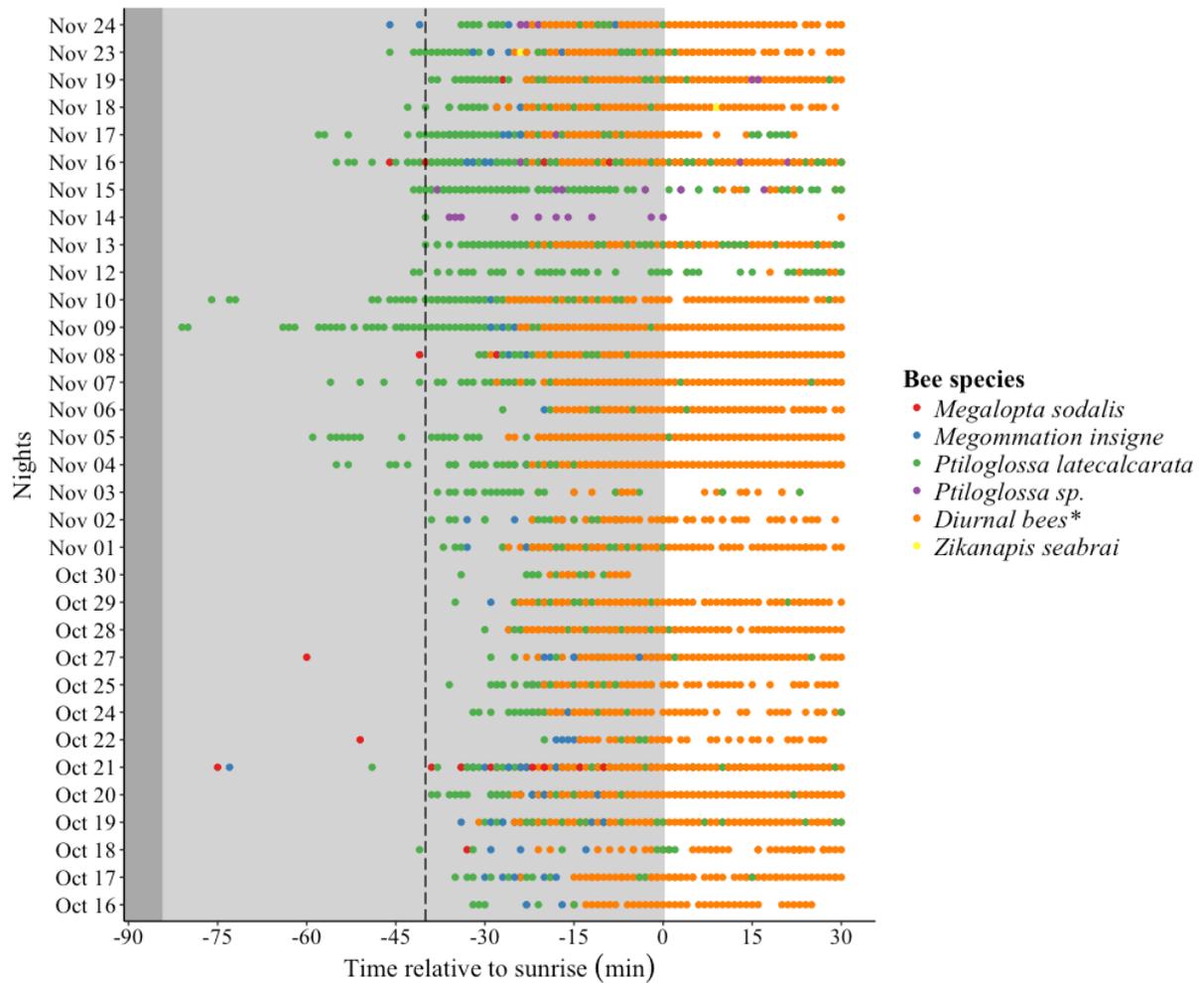
**Figure S5.** Distribution of visitation rate (visits/flower/min) from crepuscular bees in cambuci flowers during twilight aggregated in intervals of 5 min. Boxes delimit where 50% of the values are concentrated, horizontal lines indicate the median (all of them are zero), and dots represent outliers. Background colors represent night (dark grey), twilight (light grey) and daytime (white). Sunrise time (0 in the axis-x) varied between 6:29 a.m. (Brazilian Summer Time, BST) in the first sampled twilight (Oct 17) to 6:09 a.m. (BST) in the last twilight (Nov 24).



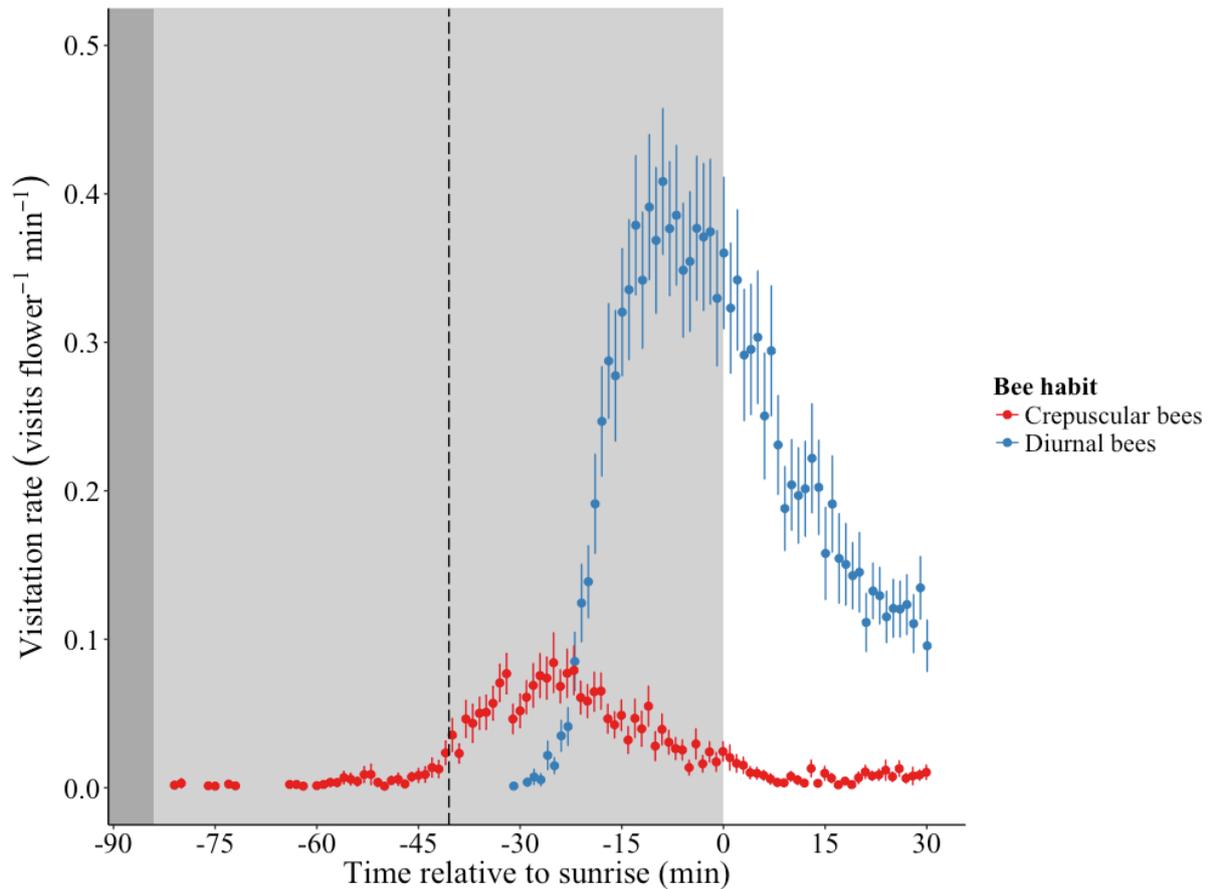
**Figure S6.** Foraging activity ranges of crepuscular bees in cambuci flowers along twilights discriminated per species. Each dot represents the time where bees were visiting cambuci in each twilight. The vertical dashed line marks the approximate half of the twilight. Background colors represent night (dark grey), twilight (light grey) and daytime (white). Sunrise time (0 in the axis-x) varied between 6:29 a.m. (Brazilian Summer Time, BST) in the first sampled twilight (October 17) to 6:09 a.m (BST) in the last twilight (November 24).



**Figure S7.** Visitation rate (visits/flower/min) from five crepuscular bees in cambuci flowers during twilight period and the beginning of the morning. Dots are average values and bars are standard errors of the mean. Background colors represent true night (dark grey), twilight (light grey) and daytime (white). The vertical dashed line marks the approximate half of the twilight. Sunrise time (0 in the axis-x) varied between 6:29 a.m. (Brazilian Summer Time, BST) in the first sampled twilight (Oct 17) to 6:09 a.m. (BST) in the last twilight (Nov 24).



**Figure S8.** Foraging activity ranges of crepuscular and diurnal bees in cambuci flowers along twilights discriminated per species. Each dot represents the time where bees were visiting cambuci in each twilight. The vertical dashed line marks the approximate half of the twilight. Background colors represent night (dark grey), twilight (light grey) and daytime (white). Sunrise time (0 in the axis-x) varied between 6:29 a.m. (Brazilian Summer Time, BST) in the first sampled twilight (October 17) to 6:09 a.m (BST) in the last twilight (November 24). \* Diurnal bees include only social bees, mostly *Apis mellifera* (99.65% of the diurnal visits/flower/min).



**Figure S9.** Visitation rate (visits/flower/min) from crepuscular and diurnal bees in cambuci flowers during twilight period and the beginning of the morning. Dots are average values and bars are standard errors of the mean. Background colors represent true night (dark grey), twilight (light grey) and daytime (white). The vertical dashed line marks the approximate half of the twilight. Sunrise time (0 in the axis-x) varied between 6:29 a.m. (Brazilian Summer Time, BST) in the first sampled twilight (Oct 17) to 6:09 a.m. (BST) in the last twilight (Nov 24). Diurnal bees include only social bees, mostly *Apis mellifera* (99.65% of the diurnal visits/flower/min).