



UNIVERSIDADE FEDERAL DE MINAS
GERAIS

Instituto de Ciências Biológicas

Programa de Pós-graduação em Zoologia



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**VISUAL ADAPTATIONS OF CREPUSCULAR BEES AND THEIR INTERACTIONS
WITH FLOWERS OF A BAT-POLLINATED TREE SPECIES**

Belo Horizonte

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Priscila de Cássia Souza Araújo

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INTERACTIONS WITH FLOWERS OF A BAT-POLLINATED TREE SPECIES**

Tese apresentada ao Programa de Pós-Graduação em Zoologia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Zoologia.

Orientador: Clemens Schindwein

Coorientador: Theo Mota

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ATA DE DEFESA DE TESE

PRISCILA DE CÁSSIA SOUZA ARAÚJO

Ao vigésimo quinto dia do mês de novembro do ano de dois mil e vinte e um, às quatorze horas, realizou-se, por webconferência, a defesa de Doutorado da Pós-Graduação em Zoologia, de autoria da Doutoranda **Priscila de Cássia Souza Araújo** intitulada: “Visual adaptations of crepuscular bees and their interactions with flowers of a bat-pollinated tree species”. Abrindo a sessão, o Presidente da Comissão, Prof. Dr. Clemens Peter Schlindwein, após dar a conhecer aos presentes o teor das Normas Regulamentares do Trabalho Final, passou a palavra para a candidata para apresentação de seu trabalho. Esteve presente a Banca Examinadora composta pelos membros: Guaraci Duran Ribeiro, Isabel Alves dos Santos, Jerome Paul Armand Laurent Baron, Reisla Silva de Oliveira, e demais convidados. Seguiu-se a arguição pelos examinadores, com a respectiva defesa da candidata. Após a arguição, apenas os Srs. Examinadores permaneceram na sala para avaliação e deliberação acerca do resultado final, a saber: o trabalho foi APROVADO SEM ALTERAÇÕES

Belo Horizonte, 25 de novembro de 2021

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RESUMO

Ao amanhecer e anoitecer, quando os níveis de luz no ambiente são menores do que o dia, abelhas crepusculares saem dos seus ninhos em busca de flores. Essas abelhas exploram flores de antese noturna, como as polinizadas por morcegos, ou flores de antese diurna com o início da abertura floral durante o crepúsculo, como as flores polinizadas por abelhas. Alguns estudos sugerem que as abelhas crepusculares se beneficiam de coletar recurso nestas flores antes da chegada dos competidores, mas isso ainda não foi demonstrado quantitativamente. Ademais, pouco é conhecido sobre o papel das pistas florais, em especial das pistas visuais, na busca por flores por essas abelhas. Apesar disso, as abelhas crepusculares possuem várias adaptações no sistema visual como ocelos, olhos e facetas grandes, que aumentam a sensibilidade a luz permitindo, assim, navegar durante o crepúsculo. Nesse sentido, embora estas adaptações estejam relacionadas ao hábito crepuscular, ainda não está claro se elas também estão relacionadas ao tamanho corporal, como é descrito para abelhas diurnas. Por fim, ainda que tenha sido sugerido que abelhas crepusculares pudessem usar a fototaxia para navegação, até então não foi descrito o comportamento fototático destas abelhas. Assim, os trabalhos que compõem esta tese tiveram como objetivo (i) avaliar a eficiência de remoção de pólen das abelhas crepusculares e comparar com outros grupos de visitantes florais em flores quiropterófilas, além de (ii) descrever as pistas florais usadas pelas abelhas durante o forrageamento sob baixa luz. Para desenvolver ambos os objetivos escolhemos como modelo a espécie vegetal quiropterófila *Pseudobombax longiflorum* (Malvaceae). Também (iii) descrever e comparar a relação do tamanho corporal no diâmetro das estruturas que compõem o sistema visual das abelhas crepusculares e diurnas. Por fim, (iv) descrever a resposta fototática das abelhas crepusculares a diferentes comprimentos de onda e intensidade de luz. Durante o crepúsculo, as abelhas crepusculares exploram as flores quiropterófilas ricas em pólen sem a presença de competidores. Nessas visitas, elas coletam muito mais pólen por minuto do que os outros grupos de visitantes florais ao longo de toda a antese. Este resultado indica que é vantajoso para essas abelhas explorarem as flores durante os curtos períodos no crepúsculo. Para encontrar essas flores, as abelhas crepusculares usam os odores e cores florais como pistas. O uso de ambas as pistas florais pelas abelhas crepusculares pode facilitar a busca pelas flores, principalmente nos períodos em que a intensidade de luz no ambiente é extremamente baixa. Quanto ao sistema visual das abelhas, os resultados mostram que o diâmetro das estruturas visuais está relacionado ao padrão temporal e tamanho corporal. Assim, as abelhas com hábito crepuscular possuem um maior diâmetro de ocelos, olhos, facetas, e menor densidade de faceta

por área de olho. Isso indica que o sistema visual desses insetos investe em sensibilidade à luz. O contrário foi observado nas abelhas com hábito diurno, o que sugere um maior investimento em acuidade visual. Em relação ao tamanho, embora os ocelos, olhos e facetas dorsal e ventral estejam correlacionados com o tamanho corporal das abelhas crepusculares, as espécies menores possuem facetas frontais tão grandes quanto às maiores. Uma explicação seria que, devido à importância da região frontal dos olhos durante o forrageamento, ao longo da história evolutiva das abelhas crepusculares, foram selecionados indivíduos pequenos, porém com grandes facetas frontais. Essa característica aumenta a sensibilidade à luz da região permitindo, assim, às abelhas pequenas voarem nos horários com baixa luminosidade. Por fim, diferente do que é descrito na literatura para abelhas diurnas, as abelhas crepusculares nem sempre são atraídas à luz. Quando a resposta fototática é desencadeada, ela é mais forte para estímulos que compreendem a região UV do espectro de luz do que azul e verde. Além disso, a intensidade luminosa não afeta o comportamento fototático, provavelmente porque os olhos das abelhas crepusculares possuem alta sensibilidade à luz.

PALAVRAS-CHAVE: competição polínica, fluxo polínico, pistas florais, ocelos, olhos compostos, fototaxia positiva, *Megalopta*, *Ptiloglossa*, abelha noturna.

ABSTRACT

Crepuscular bees visit flowers only at dawn and dusk. During these periods, the light levels in the environment are many orders of magnitude dimmer than sunlight. While foraging, these bees exploit nocturnal flowers, such as bat-pollinated species, or diurnal flowers with the beginning of the floral opening in the twilight, such as bee-pollinated species. Some studies suggest that crepuscular bees benefit from collecting resources on these flowers before the arrival of competitors, but this has not yet been demonstrated quantitatively. Also, little is known about the role of floral cues during the foraging of crepuscular bees, especially visual ones. Despite this, crepuscular bees have several adaptations in the visual system, such as large ocelli, eyes, and facets, which increase sensitivity to light, thus allowing them to navigate during dim-light conditions. Although these adaptations are related to the crepuscular habit, it is still unclear whether the structures of visual system are also related to body size, as in diurnal bees. Finally, although it has been suggested that crepuscular bees use phototaxis to navigate, the phototactic behavior of these bees has not been described so far. Therefore, the objectives of the chapters of this thesis were (i) to evaluate the efficiency of pollen collection of crepuscular bees and compare with other groups of floral visitors in chiropterophilous flowers and (ii) describe the floral cues used by bees during foraging in low light. To develop both objectives, we chose as a model the chiropterophilous plant species *Pseudobombax longiflorum* (Malvaceae). (iii) Also, to describe and compare the influence of body size on the diameter of the visual system structures in crepuscular and diurnal bees. Finally, (iv) to describe the phototactic response of crepuscular bees to different wavelengths and light intensity. During twilight, crepuscular bees exploit the chiropterophilous flowers when these are still rich in pollen and without the presence of competitors. On these visits, they collect much more pollen per minute than the other groups of floral visitors throughout the anthesis. This result indicates that it is advantageous for these bees to explore the flowers during the brief periods of twilight. Furthermore, to find these flowers, crepuscular bees use the floral odors and colors as cues. The use of both floral cues by crepuscular bees can facilitate the search for flowers, especially in periods when the light intensity in the environment is extremely low. About the visual system, we found that the diameter of the visual structures of bees are related by temporal patterns and body size. Therefore, bees with crepuscular habit have a larger diameter of ocelli, eyes, facets, and lower facet density per eye area. This indicates that the visual system of these insects invests in light sensitivity. The opposite was observed in bees with a diurnal habit, which suggests a greater investment in visual acuity. Regarding the size, although ocelli, eyes and dorsal and

ventral facets are correlated with the body size of crepuscular bees, smaller species have frontal facets as large as the bigger ones. An explanation for this could be that, due to the importance of the frontal region of the eyes during foraging, throughout the evolutionary history of crepuscular bees, small individuals were selected, but with large frontal facets. This characteristic increases light sensitivity in the region, allowing small bees to fly in periods of low light. Finally, unlike what is described in the literature for diurnal bees, crepuscular bees are not always attracted to light. When the phototactic response is triggered, it is stronger for stimuli that comprise the UV region of the light spectrum than blue and green. Furthermore, light intensity does not affect phototactic behavior, probably because crepuscular bee eyes have high light sensitivity.

KEYWORDS: pollen competition, pollen flow, floral cues, ocelli, compound eyes, positive phototaxy, *Megalopta*, *Ptiloglossa*, nocturnal bee.

SUMÁRIO

INTRODUÇÃO GERAL	13
REFERÊNCIAS	16
CAPÍTULO I.....	20
THE ADVANTAGES OF BEING CREPUSCULAR FOR BEES: MAJOR POLLEN GAIN UNDER LOW COMPETITION DURING THE BRIEF TWILIGHT PERIOD	20
ABSTRACT	21
1. INTRODUCTION	22
2. MATERIAL AND METHODS.....	23
3. RESULTS.....	28
4. DISCUSSION.....	32
5. REFERENCES	37
FIGURES AND TABLES.....	45
SUPPORTING INFORMATION	51
CAPÍTULO II	52
PISTAS VISUAIS E OLFATIVAS UTILIZADAS NO CREPÚSCULO POR ABELHAS EM BUSCA DE RECURSOS EM FLORES QUIROPTERÓFILAS	52
RESUMO	53
1. INTRODUÇÃO.....	55
2. MATERIAIS E MÉTODOS.....	57
3. RESULTADOS	63
4. DISCUSSÃO.....	72
5. REFERÊNCIAS	75
CAPÍTULO III.....	83
BODY SIZE AND THE ARCHITECTURE OF VISUAL ORGANS IN CREPUSCULAR AND DIURNAL PHYLOGENETICALLY RELATED BEES.....	83
ABSTRACT	84
1. INTRODUCTION	85
2. MATERIAL AND METHODS.....	88
3. RESULTS.....	91
4. DISCUSSION.....	99
5. REFERENCES	103
CAPÍTULO IV	110
SPECTRAL SENSITIVITY OF THE POSITIVE PHOTOTAXIS IN CREPUSCULAR BEES	110

ABSTRACT	111
1. INTRODUCTION	112
2. MATERIAL AND METHODS.....	113
3. RESULTS.....	119
4. DISCUSSION.....	123
5. REFERENCES	126
CONCLUSÃO GERAL	131

1 INTRODUÇÃO GERAL

2 Abelhas, em geral, são insetos de hábito diurno, mas algumas espécies evoluíram
3 a capacidade de voar em períodos com baixa intensidade de luz (Wcislo e Tierney, 2009).
4 Esse comportamento surgiu 19 vezes de forma independente ao longo da história
5 evolutiva do grupo e está presente em quatro das sete famílias, Colletidae, Andrenidae,
6 Halictidae e Apidae (Wscilo e Tierney, 2009). As abelhas que forrageiam exclusivamente
7 nos horários de baixa luminosidade são conhecidas como abelhas crepusculares, noturnas,
8 matinais ou vespertinas (Linsley, 1960; Linsley e Cazier, 1970; Warrant et al., 2004;
9 Kelber et al., 2006; Wscilo e Tierney, 2009). Embora esses nomes sejam frequentemente
10 usados como sinônimos, adotamos o termo abelha crepuscular, pois as abelhas estudadas
11 nesta tese têm sua atividade de voo concentrada entre o crepúsculo náutico e o nascer do
12 sol e entre o pôr do sol e o crepúsculo náutico (Kelber et al., 2006; Liporoni et al., 2020).
13 Nesse período, várias espécies representantes dos gêneros *Megalopta* (Halictidae:
14 Augochlorini), *Megommation* (Halictidae: Augochlorini), *Ptiloglossa*
15 (Colletidae:Caupolicanini) e *Zikanapis* (Colletidae:Caupolicanini) visitam as flores
16 (Siqueira et al., 2018; Krug et al., 2018; Liporoni et al., 2020; Araujo et al., 2020). No
17 entanto, embora a visita das abelhas às flores se concentre no crepúsculo, é comum
18 observar a visita das abelhas *Ptiloglossa* no início da manhã (Linsley e Cazier, 1970;
19 Liporoni et al., 2020; Araujo et al., 2020; Araújo et al., 2021). Além dos hábitos diurno e
20 crepuscular, algumas espécies são consideradas crepusculares facultativas, como
21 representantes do gênero *Caupolicana* (Colletidae:Caupolicanini) (Wscilo e Tierney,
22 2009). Essas abelhas forrageiam no crepúsculo, mas são capazes de estender as suas
23 atividades para algumas horas após o nascer do sol, ou antes do pôr do sol (Linsley e
24 Cazier, 1963; Linsley e Cazier, 1970). Por fim, alguns representantes são considerados
25 noturnos, como a abelhas *Xylocopa tranquebarica* (Apidae: Xilocopini) e *Lasioglossum*
26 (*Sphecodogastra*) *texana* (Halictidae: Augochlorini) (Somanathan et al. 2008;
27 Somanathan et al. 2009; Kerfoot, 1967a). Ambas as espécies são capazes de buscar por
28 flores ao longo da noite (Kerfoot, 1967a, Somanathan et al., 2020), contudo, *L. texana* só
29 é capaz de fazer isso em noites de luar (Kerfoot, 1967a).

30 Duas hipóteses foram sugeridas para explicar a transição evolutiva do nicho
31 diurno para o crepuscular pelas abelhas: a primeira está associada ao menor risco de
32 predação e parasitismo nos ninhos e a segunda é referente à redução de competidores por
33 recursos florais (Wcislo et al., 2004). Dados de parasitismo de ninho de abelhas são

34 escassos na literatura, mas o estudo de Wcislo et al., (2004) indica que os ninhos de
35 *Megalopta* são menos parasitados quando comparados aos de outras abelhas diurnas. Já
36 em relação à segunda hipótese, os estudos mostram que abelhas crepusculares são capazes
37 de explorar flores de antese noturna, como as quiropterófilas, ainda no início da antese,
38 quando o recurso ainda não foi coletado por nenhum outro grupo de visitante floral, ou
39 no amanhecer, antes dos visitantes diurnos (Hopkins et al., 2000; Somanathan e Borges,
40 2001; Wcislo et al., 2004; Smith et al., 2012; Araujo et al., 2020). Ademais, as abelhas
41 crepusculares são as primeiras a explorar as flores melitófilas que possuem o início da
42 antese no crepúsculo (Krug et al., 2015; Cordeiro et al., 2017; Siqueira et al., 2018).
43 Porém, ainda não foi demonstrado se a quantidade de recurso floral coletada no curto
44 período de atividade dessas abelhas sem a presença de um competidor, de fato, é uma
45 vantagem. Baseado nisso, no primeiro capítulo demonstramos quantitativamente a
46 eficiência de coleta de pólen pelas abelhas crepusculares.

47 As abelhas crepusculares usam os odores das flores melitófilas para encontrá-las
48 durante o amanhecer. Essas abelhas são atraídas pelos compostos majoritários das flores
49 de *Campomanesia phaea*, *Paulinia cupana* e perfumes usados em armadilha para capturar
50 machos de Euglossini (Knoll e Santos, 2012; Carvalho et al., 2012; Cordeiro et al., 2017;
51 Krug et al., 2018; Martinez-Martinez et al., 2021). Contudo, os voláteis majoritários
52 produzidos por flores quiropterófilas diferem dos compostos descritos em flores
53 melitófilas, por exemplo os compostos de enxofre (von Helversen et al., 2000). Assim,
54 não se sabe se as abelhas crepusculares são capazes de usar o cheiro desagradável das
55 flores quiropterófilas como pista durante o forrageamento. Além disso, embora alguns
56 estudos descrevam a importância de pistas visuais durante a navegação e reconhecimento
57 da entrada do ninho por abelhas crepusculares (Warrant et al., 2004; Chaib et al., 2020),
58 não se sabe ainda se elas também usam pistas visuais para reconhecer as flores. Dessa
59 forma, o segundo capítulo desta tese descreve o papel das pistas olfativas e visuais na
60 busca por flores quiropterófilas pelas abelhas crepusculares.

61 Buscar por flores em períodos de baixa intensidade de luz, no entanto, não é uma
62 tarefa trivial. O sistema visual das abelhas crepusculares precisa lidar com problemas
63 como a baixa quantidade de fótons de luz no ambiente e o ruído fisiológico presente no
64 fotorreceptor (Warrant, 2017). Ambos podem prejudicar o sinal visual, tornando-o pouco
65 confiável (Warrant, 2017). Contudo, os olhos compostos de aposição das abelhas
66 crepusculares possuem um conjunto de adaptações que ajudam a resolver os problemas

67 de enxergar durante a noite. Seus ocelos, olhos e as facetas que compõem os olhos
68 compostos são grandes em relação ao tamanho corporal (Kerfoot, 1967b; Jander e Jander,
69 2002; Warrant et al., 2004; Greiner et al., 2004 a). Além disso, os rabdomas - conjunto
70 de células fotossensíveis fusionadas - são largos e longos (Warrant et al., 2004; Greiner
71 et al., 2004a). Essas alterações no sistema visual maximiza a captura de fótons,
72 aumentando, assim, a sensibilidade a luz (Jander e Jander, 2002; Warrant et al., 2004;
73 Greiner et al., 2004 a). Dessa forma, os olhos compostos de *Megalopta genalis*, abelha
74 crepuscular mais estudada, é 28 vezes mais sensível à luz do que o de *Apis mellifera*
75 (Warrant et al., 2004). Além disso, os fotorreceptores das *Megalopta genalis* trocam a
76 capacidade de informação por ganho na sensibilidade à luz (Frederiksen et al., 2008). Por
77 fim, na lâmina, primeiro gânglio ótico, os interneurônios possuem extensas arborizações
78 laterais (Greiner et al., 2004b). Devido a esses achados anatômicos, sugere-se que as *M.*
79 *genalis* sejam capazes de fazer a soma neural de fótons no espaço (Greiner et al., 2004b).
80 Todavia, apesar de todos esses trabalhos descreverem as adaptações morfológicas,
81 fisiológicas e anatômicas presentes no sistema visual das *M. genalis*, ainda não está claro
82 qual a relação entre o tamanho corporal e as estruturas que compõem o sistema visual das
83 abelhas crepusculares. Visando entender essa relação, bem como descrever e comparar a
84 morfologia do sistema visual com outros grupos de abelhas crepusculares e diurnas
85 relacionadas filogeneticamente, foi construído o capítulo 3 desta tese.

86 Outra questão ainda não explorada nas abelhas crepusculares, e pouco
87 compreendida nas abelhas diurnas, é em relação ao comportamento fototático. A fototaxia
88 é um comportamento estereotipado no qual o organismo é atraído ou repelido pelo
89 estímulo luminoso (Jander, 1963). As abelhas possuem fototaxia positiva, que é
90 influenciada tanto pela intensidade de luz, como também pelo comprimento de onda
91 (Kaiser et al. 1977; Menzel e Greggers, 1985; Nouvin e Galizia, 2020). É sugerido que a
92 resposta à luz por essas abelhas esteja relacionada ao comportamento de escape e de
93 orientação visual (Menzel e Greggers, 1985; Nouvin e Galizia, 2020). Embora até o
94 momento ainda não tenha sido descrito o comportamento fototático das abelhas
95 crepusculares, Kelber et al., (2006) sugeriu que *M. genalis* usa a iluminação do dossel,
96 que é maior do que a iluminação no interior da floresta, para navegar no crepúsculo,
97 usando assim a fototaxia. Dessa forma, o quarto capítulo desta tese descreve o
98 comportamento fototático das abelhas crepusculares aos diferentes comprimentos de
99 onda e intensidade de luz.

100

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CAPÍTULO I**THE ADVANTAGES OF BEING CREPUSCULAR FOR BEES: MAJOR
POLLEN GAIN UNDER LOW COMPETITION DURING THE BRIEF
TWILIGHT PERIOD ***197
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218

219 **ABSTRACT**

220 The shift in flight activity from daylight to twilight in crepuscular bees is assumed to have
221 evolved to escape competitors, but quantitative confirmation of this hypothesis has never
222 been clearly demonstrated. *Pseudobombax longiflorum* is a chiropterophilous plant with
223 flowers presenting large amounts of pollen throughout anthesis, thus attracting not only
224 nocturnal visitors, but also crepuscular and diurnal bees. In this dynamic system, the
225 fraction of pollen that flows to different visitors and the putative role of bees in pollination
226 remain unknown. Here, we analysed floral biology, the frequency of visitors in periods
227 with different light intensities and the pollen removal rate by each visitor group. A
228 pollinator exclusion experiment showed that bees are not pollinators of *P. longiflorum*,
229 although they collected >60% of the pollen of their flowers. Crepuscular bees gained the
230 greatest amount of pollen in the few minutes they foraged without either nocturnal or
231 diurnal competitors, thus confirming the advantage of foraging under low light. During
232 the short twilight period, these bees foraged alone, and removed 26.5 and 15 times more
233 pollen per minute than nocturnal and diurnal visitors, respectively. Therefore, pollen
234 removal by crepuscular bees is particularly efficient when foraging in the brief period
235 when competitors are absent.

236

237 **KEYWORDS:** floral resource collection - nocturnal bees - pollen competition - pollen
238 fate – pollination – *Pseudobombax* - *Ptiloglossa*.

239 1. INTRODUCTION

240 The flight activity of crepuscular bees is restricted to the short dim-light period at
241 dusk and dawn (Linsley, 1958; Hurd & Linsley, 1964, Michener, 1966; Janzen, 1968;
242 Kelber et al. 2006; Somanathan et al., 2009; Cordeiro et al., 2017; Liporoni et al., 2020).
243 The ability to fly during twilight is limited by the sensitivity of the visual system to light
244 (Kelber et al., 2006). Due to physiological adaptations of their compound eyes and ocelli
245 (Kerfoot, 1967; Warrant et al., 2004; Greiner et al., 2004), crepuscular bees are suggested
246 to benefit from exploiting untouched pollen in flowers that open at dusk or before dawn,
247 prior to the arrival of diurnal competitors (Cordeiro et al., 2017; Siqueira et al., 2018;
248 Araujo et al., 2020; Liporoni et al., 2020). It is assumed that less competition for floral
249 rewards in these periods might have driven the evolution of the switch to low light
250 foraging in bees (Wcislo et al., 2004).

251 In plant species adapted to nocturnal pollinators, these bees might be able to use
252 flowers that open at or near dusk to obtain floral resources before the flight activity of
253 nocturnal flower visitors, and at dawn to be the first to exploit the pollen and nectar
254 leftovers of the nocturnal pollinators (Araujo et al., 2020). In these flowers, the flight
255 activity of crepuscular bees in the early morning overlaps with that of numerous diurnal
256 bee species (Araujo et al., 2020), the period in which competition increases dramatically.
257 However, there are no quantitative data that demonstrate whether crepuscular bees really
258 benefit in terms of pollen collection on their host plants through their ability to fly in dim-
259 light conditions in contrast to their diurnal bee competitors.

260 Pollen of several bat-pollinated species has been found in brood cell provisions or
261 in scopa pollen loads of crepuscular bees (Roulston, 1997; Wcislo et al., 2004; Smith et
262 al., 2012; Smith et al., 2017; Araujo et al., 2020). Due to the large amounts of resources
263 that generally remain in these flowers after the nocturnal visit of bats, crepuscular bees
264 and several groups of diurnal animals have repeatedly been observed to visit the flowers
265 in the morning, such as hummingbirds, butterflies, and diurnal bees (Baker, 1961; Alcorn
266 et al., 1961; Sahley, 1996; Sazima & Sazima, 1978; Schmidt & Buchmann 1986; Slauson
267 2000; Muchhala, 2003; Ibarra-Cerdeña et al., 2005; Rivera-Marchand & Ackerman,
268 2006; Muchhala, 2007; Lassen et al., 2012; 2017; Queiroz et al., 2016; Wayo et al., 2018;
269 Araujo et al., 2020; Rocha et al., 2020). Some studies assigned those visitor groups a
270 certain occasional contribution to the pollination of bat-pollinated flowers (Sazima &

271 Sazima, 1978; Ibarra-Cerdeña et al., 2005; Rivera-Marchand & Ackerman, 2006; Lassen
272 et al., 2012, Hernández-Montero & Sosa, 2016).

273 To obtain insights into the profitability of bat-pollinated flowers for bees in terms
274 of pollen gain, we chose a representative of the neotropical tree genus *Pseudobombax*
275 Dugand (Malvaceae, Bombacoideae), which is a classic example for chiropterophilous
276 blossoms in the Neotropics (Knuth, 1904; Vogel, 1958, 1969ab; Heithaus et al., 1975;
277 Dobat & Peikert-Holle, 1985; Eguiarte et al., 1987; Fischer et al., 1992; Gottsberger &
278 Silberbauer-Gottsberger, 2006), as study model: *Pseudobombax longiflorum* (Mart. et
279 Zucc.) A. Robyns. Previously, we have recorded frequent crepuscular and diurnal bee
280 visits in the flowers of this species and therefore wanted to answer the following
281 questions: (i) What is the role of bees as pollinators of *P. longiflorum*? (ii) What is the
282 overall pollen fate and the efficiency of pollen collection by crepuscular bees? (iii) How
283 does light intensity relate to the presence of floral visitors and the pollen amount acquired
284 by them? To answer these questions, we analyzed floral traits and anthesis, quantified the
285 pollen resources per flower, determined the frequency of bee visitors, conducted a
286 pollinator exclusion experiment to determine the role of bees as pollinators, measured the
287 absolute light intensity during the phases of flower visiting of the different animals, and
288 quantified the pollen decrease per flower in these visitor phases.

289

290 **2. MATERIAL AND METHODS**

291 **2.1 Study area**

292 The study was conducted during three flowering periods, from May to July 2018–
293 2020, in an area of Cerrado vegetation on a limestone outcrop situated at Serra do Cipó
294 (19°18'40.7"S 43°36'42.2"W), Minas Gerais, Brazil. The dry season during the cooler
295 winter months (July–September) is well separated from the rainy season in the summer
296 (Giulietti et al., 1987).

297 **2.2 Study species**

298 The genus *Pseudobombax* (Malvaceae, Bombacoideae) contains 29 species of
299 trees with self-incompatible and robust white brush blossoms with numerous long
300 stamens (Carvalho-Sobrinho & Queiroz, 2010). The flowers have nocturnal anthesis,
301 produce large quantities of pollen and nectar, and emit a strong scent during the night. As

302 pollinating bat species have been reported: *Artibeus jamaicensis* Leach, 1821, *A. phaeotis*
303 (Miller, 1902), *Carollia perspicillata* (Linnaeus, 1758), *Choeronycteris mexicana*
304 Tschudi, 1844, *Glossophaga leachii* (Gray, 1844), *Glossophaga soricina* (Pallas, 1766),
305 *Lonchophylla dekeyseri* Taddei, Vizotto & Sazima, 1983, *Phyllostomus hastatus* (Pallas,
306 1767), *Phyllostomus discolor* (Wagner, 1843), and *Sturnira lilium* (É. Geoffroy St.-
307 Hilaire, 1810) (Heithaus et al., 1975; Eguiarte et al., 1987; Fischer et al., 1992; Silva &
308 Peracchi, 1995; Gribel & Gibbs, 2002; Pequeno et al., 2016; Peterle et al., 2007). Small
309 nocturnal marsupials have also been reported as pollinators of a species of the genus
310 (Eguiarte, 1987; Gribel, 1988).

311 *Pseudobombax longiflorum* is a tree up to 25 m in height that frequently occurs
312 on limestone outcrops and sheds foliage in the dry season, which is also the flowering
313 period (Lorenzi, 1992; Alves da Silva & Scariot, 2004). At the study site in the Serra do
314 Cipó, the trees flower from May to July (Esteves, 1992). Bats of *Lonchophylla dekeyseri*
315 have been cited as likely pollinators (Coelho & Marinho-Filho, 2002). A voucher
316 specimen of *Pseudobombax longiflorum* was deposited in BHCB Herbarium at Federal
317 University of Minas Gerais (UFMG), Belo Horizonte, Brazil.

318 **2.3 Flower morphology and anthesis**

319 During 12 non-consecutive days, we recorded the opening time and the
320 senescence in 160 flowers of seven trees. We applied droplets of hydrogen peroxide
321 (H₂O₂) to the stigma surface at the beginning of anthesis and in the morning (n = 12) to
322 check for stigma receptivity (Dafni et al., 2005) and determined the time of anther
323 dehiscence by monitoring them with a hand-held magnifying glass. The lengths of styles
324 and stamens were measured with a digital caliper. The numbers of stamens and ovules
325 were counted in 12 flowers from seven trees. We then calculated the standard deviation
326 of the number of stamens and ovules from these 12 flowers

327 **2.4 Floral resources**

328 To determine the number of pollen grains per flower, we fixed a total of 15 flower
329 buds in pre-anthesis in 70% ethanol taken from seven plant individuals. We removed five
330 stamens of different positions (from outer to central) of each flower and macerated each
331 anther separately in Eppendorf tubes with 0.5 ml of a 3:1 mixture of lactic acid and
332 glycerin. After homogenization in a vortex stirrer, an aliquot per anther was removed
333 (0.01 ml), the pollen grains were counted under a stereomicroscope, and the total number

334 of pollen grains per anther was calculated by multiplying the counted grains per aliquot
335 with the suspension volume. The number of pollen grains per flower was estimated
336 multiplying the mean number of grains per anther ($n = 5$ per specimen) with the number
337 of stamens per flower.

338 Nectar volume was measured soon after flower opening (18:20 h) with 1-ml
339 syringes in six flowers of different trees, and the concentration of sugars was measured
340 in eight flowers using a refractometer (Instrutherm, RT-82).

341 **2.5 Flower visitors**

342 During dusk (17:30–18:00 h) and dawn (5:30–6:00 h) and in the early morning
343 (6:00–7:00 h), we observed the crepuscular and diurnal flower visitors of *P. longiflorum*,
344 which were exclusively bees. Bees were sampled with entomological nets for 11 non-
345 consecutive days in two flowering seasons (2019 and 2020). The bees were pinned,
346 labeled, identified, and deposited in the Entomological Collection of the Universidade
347 Federal de Minas Gerais.

348 The frequency of flower visitors was determined in intervals of 10 minutes during
349 dusk and dawn. The frequency at dusk was quantified for 8 days on 16 flowers. In
350 addition, we also determined the frequency of floral bud inspection by bees that occurred
351 only at dusk. At dawn, the frequency of visitors was measured for 11 days in 22 flowers.

352 During the flower visits, we observed whether the bees sought for pollen and/or
353 nectar. In addition, we noted if the bees contacted the stigma during the visits.

354 **Analyses of pollen loads of crepuscular and diurnal bees**

355 To obtain information on the flower constancy of bees on their foraging flights to
356 *P. longiflorum*, we removed scopa pollen loads of bee females sampled at dawn and in
357 the morning, mixed the loads in 70% ethanol and transferred a sample to a microscope
358 slide. The samples were imbedded in gelatin, heated in an alcohol flame, covered with
359 coverslips, and sealed with paraffin (Louveaux et al., 1978; Schlindwein et al., 2009).
360 The pollen grains of each slide were identified under a light microscope, and the relative
361 frequencies of the different morpho-types were determined. We counted at least 500
362 pollen grains per slide.

363 **2.6 Solar irradiance**

364 We measured the absolute solar irradiance ($\mu\text{W}/\text{cm}^2/\text{nm}$) during both twilight
365 periods and in the morning to associate the activity period of flower-visiting bees with
366 the light intensity. These measurements were performed at the same time intervals in
367 which the frequencies of bees were monitored. We used a spectrophotometer
368 (USB2000+UV-VIS-ES, Ocean Optics, Dunedin, FL, USA) radiometrically calibrated
369 by means of a deuterium/tungsten light source (DH-2000-BAL, 220–1050 nm, Ocean
370 Optics). Absolute solar irradiance from 300 to 800 nm was measured using an optical
371 fiber (QP600-2-UV-VIS, Ocean Optics) coupled to a cosine corrector with Spectralon
372 diffusing material (CC-3-UV-S, Ocean Optics). The software SpectraSuite (Ocean
373 Optics) was used for acquisition and analysis of spectral curves. The reference
374 wavelength for comparing solar irradiance values among different time intervals was 450
375 nm.

376 **2.7 Visitor exclusion experiment**

377 To know whether bee visits to flowers of *P. longiflorum* contribute to fruit set,
378 two treatments were established: (i) bee pollination – flowers were bagged after dusk and
379 maintained like this during the night until dawn (05:00 h), when the bags were removed
380 to again allow flower visits ($n = 32$); (ii) natural pollination – unbagged flowers were
381 maintained accessible to flower visitors throughout anthesis ($n = 32$). We determined fruit
382 and seed set in each treatment.

383 **2.8 Pollen fate**

384 To determine the pollen fate of *P. longiflorum* in the field, we individually marked
385 23 flowers; 16 flowers were visited by crepuscular bees only at dawn and 7 flowers by
386 crepuscular bees at dusk and dawn. All flowers were accessible to visitors throughout
387 anthesis. We counted the pollen grains per anther at different moments of anthesis: **(0)**
388 total number of pollen per anther (see details above in “*Floral resources*”); **(1)** number
389 of pollen per anther after crepuscular bee visits during dusk ($n = 7$) (18:00 h); **(2)** number
390 of pollen per anther before dawn and the first bee visits (5:30 h); until this time of anthesis,
391 flowers were accessible to nocturnal flower-visiting animals; **(3)** number of pollen per
392 anther after exclusive crepuscular bee visits at dawn, before the first visits of diurnal bees
393 (5:50 h); **(4)** number of pollen per anther after the overlap period of crepuscular and
394 diurnal bee visits (6:20 h); **(5)** number of pollen per anther after the period of exclusive
395 diurnal bee visits (7:00 h). After this period, visits of diurnal bees no longer occurred.

396 Three anthers from different positions were removed at each sampling moment
397 from the flower and placed together inside an Eppendorf tube containing 70% ethanol. In
398 the laboratory, the ethanol was evaporated in a drying chamber at a temperature of 35°C,
399 and 0.5 ml lactic acid and glycerin at 3:1 was added to the Eppendorf tube (Lloyd, 1972).
400 The anthers were macerated, and the solution was homogenized in a vortex stirrer for 2
401 min. An aliquot of 0.01 ml was removed and transferred to a microscope slide, and all
402 pollen grains were counted under the microscope. Subsequently, we estimated the amount
403 of pollen per anther in each treatment.

404 To determine pollen fate, we calculated the amount and percentage of pollen
405 grains removed by each group of floral visitors. The percentage of pollen removed by
406 floral visitors was calculated from the total amount of pollen from the closed anthers.
407 Nocturnal visitors were considered responsible for pollen removal in the period between
408 flower-visiting crepuscular bees at dusk and 5:30 h (2) or, in the case no bee visitors
409 occurred at dusk, for the pollen removal from flower opening until 5:30 h (2). When
410 crepuscular bees visited the flowers at dusk and dawn, they were considered responsible
411 for pollen removal at dusk (1) and early dawn (3). In (4), pollen removal was performed
412 by diurnal and crepuscular bees, and in the last count at 7:00 h (5), we calculated the
413 percentage of pollen collected by the diurnal bees. At the end of anthesis, we counted the
414 amount of residual pollen grains in the anthers.

415 Moreover, we determined the number of pollen grains that adhered to the stigma
416 at the end of anthesis ($n = 15$ flowers). Each stigma was embedded in glycerin gelatin on
417 a microscope slide, covered with a coverslip, and sealed with paraffin (Schlindwein et al.,
418 2005).

419 **2.9 Data analysis**

420 To compare the fruit set after the treatments “bee pollination” and “natural
421 pollination”, we used the Chi-square test. To compare the number of inspected buds and
422 visited new flowers by bees, we used the paired t test. To compare the number of pollen
423 grains remaining in the anther after each floral visit, as well as to compare the number of
424 pollen grains collected by each floral visitor, we used General Mixed Model analysis
425 (GLMM) with Poisson and negative binomial family distribution, respectively. In both
426 analyses, the response variable was the number of pollen grains. We considered the
427 treatment as fixed effects (predictor variable) and the plant and flowers as random
428 variables. When necessary, we performed planned comparisons among the treatments,

429 using the multcomp package. All analyses were performed in the R environment (R Core
430 Team, 2020).

431

432 3. RESULTS

433 3.1 Flower morphology, floral resources, and anthesis

434 The flowers of *P. longiflorum* contained an average of $296.4 \pm (=std) 32.6$ stamens
435 ($n = 12$), with a mean length of 12.3 ± 1.9 cm ($n = 12$). The style measured on average
436 14.9 ± 1.8 cm ($n = 12$), being thus 2.6 cm longer than the stamens (Figure 1). The ovary
437 carried, on average, 154.5 ± 24.0 ovules ($n = 6$).

438 The flowers contained an average of $13,434,782 (\pm 2,339,892)$ pollen grains ($n =$
439 15), and a single anther contained $45,316 \pm 7,892$ pollen grains ($n = 75$). Thus, the pollen-
440 to-ovule ratio was 86,956:1. At the time of floral opening, the flowers contained an
441 average of 1.19 ± 0.19 ml ($n = 6$) of nectar, with a sugar concentration of $18.3\% \pm 0.5\%$
442 ($n = 8$).

443 The flowers opened within a span of 1 hour, between 17:30 h and 18:30 h (Figure
444 3A). Maximum floral opening was achieved 17.0 ± 16.3 min ($n = 10$) after the beginning
445 of the unfolding of the petals, when the adaxial surface of the petals curved backward
446 below the stamens. When the petals unfolded, all anthers were already dehisced, exposing
447 the pollen grains ($n = 12$), and the stigma was already receptive, remaining like this until
448 12:00 h of the following morning ($n = 12$). Floral senescence occurred around 16:00 h on
449 the following day, when the stamens wilted and bent downwards. Corolla and stamens
450 detached from the receptacle and fell 2 days later.

451 3.2 Flower-visiting bees

452 The flowers of *Pseudobombax longiflorum* were visited by females and males of
453 the crepuscular bees *Ptiloglossa stafuzzai* Moure, 1945 and *Ptiloglossa xanthotricha*
454 Moure, 1945, females of the carpenter bee *Xylocopa (Neoxylocopa) grisescens*
455 Lepeletier, 1841, and worker bees of *Apis mellifera* Linnaeus, 1758 and *Trigona*
456 *hyalinata* (Lepeletier, 1836) (Figure 2A-B, Table 2).

457 At dusk, we recorded several visits of females of *Ptiloglossa* to new flowers of *P.*
458 *longiflorum* until ~18:00 h and of two females of *X. grisescens* until ~17:40 h (Figure

459 3B). After the last flower visit of crepuscular *Ptiloglossa* in the evening, more than half
460 of the flowers (57%; 91 of 160 flowers) had still not started anthesis (Figure 3A).

461 We observed females of *Ptiloglossa* and *X. grisescens* flying within the crown
462 area of *P. longiflorum* already before the flowers opened. The bees flew in circles around
463 the flower buds, inspecting them without landing. We noted such bud inspection also later
464 when the first flowers had already started anthesis and in younger flower buds that would
465 open only on the following days. In 8 of the 11 observation days, bees of *Ptiloglossa*
466 visited flowers at dusk, but only on 1 day those of *X. grisescens*. Out of 103 recorded bees
467 of *Ptiloglossa* at dusk, 66 individuals (64%) inspected buds and 37 (36%) visited new
468 flowers ($t = 3.1$; $df = 4$, $p = 0.01$, Figure 2C), whereas only two (7%) of 29 observed bees
469 of *X. grisescens* visited flowers ($t = 3.3$; $df = 4$, $p = 0.01$, Figure 3C).

470 The first flower-visiting bees at dawn were *Ptiloglossa*, visiting the flowers
471 between 5:30 h and 6:20 h (Figure 2D). Carpenter bees *X. grisescens* visited flowers from
472 5:50 h to 6:20 h, honeybees from 5:50 h to 7:00 h, and the stingless bees *T. hyalinata*
473 from 6:00 h to 7:00 h. Thus, the crepuscular bees of *Ptiloglossa* were the sole flower
474 visitors over a period of 20 min, and through the following 30 min, their flower visits
475 partly overlapped with those of three recorded diurnal bee species (Figure 3D). From
476 ~06:00 h, honeybees were extraordinarily abundant flower visitors. We observed up to
477 40 honeybee individuals visiting the same flower simultaneously. Around 7:00 h, flower
478 visits ceased.

479 The sequences in which bees of the four species arrived at the flowers in the
480 morning was always the same: *Ptiloglossa*, followed by *Xylocopa*, *Apis*, and then
481 *Trigona*. Bees of *Ptiloglossa* visited the flowers when the solar irradiance was between
482 1.98×10^{-4} and $0.19 \mu\text{W}/\text{cm}^2$, whereas the diurnal bees visited the flowers only when the
483 absolute solar irradiance was greater than $2.5 \times 10^{-2} \mu\text{W}/\text{cm}^2$ (Figure 3D).

484 During flower visits, females of *Ptiloglossa* generally grabbed a set of stamens and
485 vibrated the anthers in several short buzzes. Females of *X. grisescens* scraped groups of
486 anthers with the hind legs, and workers of *A. mellifera* and *T. hyalinata* collected pollen
487 always from one anther at a time. Both crepuscular and diurnal bees visited flowers
488 mainly to collect pollen and accessed the nectar chamber to take up nectar in less than
489 20% of visits (Table 1).

490 Occasionally, females of *X. grisescens* contacted the stigmas (<19% of the flowers
 491 visited), whereas we did not record any stigma contact by bees of *Ptiloglossa*, *A.*
 492 *mellifera*, and *T. hyalinata* throughout the field study.

493 **3.3 Analyses of pollen loads of crepuscular and diurnal bees**

494 Analyses of the scopa pollen loads of 17 females of *Ptiloglossa* revealed that 15
 495 females carried pollen from *P. longiflorum*: five had pure pollen loads of this species,
 496 whereas the loads of the other individuals contained pollen from two or three further plant
 497 species. The scopa of females of *Ptiloglossa* contained pollen from *P. longiflorum*,
 498 Sapindaceae, Mimosoideae (Fabaceae), in addition to four other unidentified pollen types
 499 (Figure S1). *Pseudobombax*-pollen represented, on average, 88.2% of the scopa pollen
 500 content (n = 15).

501 The scopa pollen loads of the carpenter bees were characterized by high
 502 proportions of pollen grains from *Pseudobombax*, complemented by Sapindaceae pollen.
 503 The corbiculae of the honeybees and of *T. hyalinata* contained pure pollen loads of *P.*
 504 *longiflorum* (Figure S1).

505 **3.4 Visitor exclusion experiment**

506 Fruit set in unbagged flowers available to floral visitors throughout anthesis
 507 (control) was 44% (14 fruits), whereas flowers which were bagged during the nighttime
 508 hours but available to floral visitors at dusk and at dawn onward set only one fruit (3%)
 509 with few seeds (Table 2).

510 **3.5 Pollen fate**

511 *Flowers visited by bees only at dawn.* Most of the 23 monitored flowers were visited by
 512 crepuscular bees only at dawn (n = 16 flowers). The mean pollen content per anther
 513 decreased significantly among subsequent intervals from 17:30 h to 7:00 h ($\chi^2 =$
 514 1,411,156, df = 4, p < 0.0001; Figure 4A).

515 There was a difference in the amount of pollen grains removed by floral visitors
 516 ($\chi^2 = 57.4$, df = 3, p < 0.0001; Figure 4B) when bees visited the flowers of *Pseudobombax*
 517 *longiflorum* exclusively at dawn. While the number of pollen grains removed in the
 518 periods of nocturnal visitors (36.6%; 16,621 \pm 8,240) and crepuscular bees alone (30.8%;
 519 13,988 \pm 9,073) and in the period of crepuscular and diurnal bee overlap (28.2%; 12,793
 520 \pm 7,082) did not differ statistically (Figure 4B), the number of pollen grains removed

521 exclusively by diurnal bees (2.5%; $1,134 \pm 1,319$) was conspicuously lower when
522 compared to the pollen removed by other floral visitors (Figure 4B).

523 *Flowers visited by crepuscular bees at dusk and dawn.* At dusk, 7 out of the 23 monitored
524 flowers were visited by crepuscular bees. The mean pollen content per anther decreased
525 significantly among subsequent intervals from 17:30 h to 7:00 h ($\chi^2 = 573,712$, $df = 5$, p
526 < 0.0001 ; Figure 4C), like in flowers visited by crepuscular bees only at dawn.

527 When crepuscular bees succeeded to visit the flowers also at dusk, the amount of
528 pollen grains removed by floral visitors also differed ($\chi^2 = 12.2$, $df = 4$, $p = 0.01$; Figure
529 4D). The number of pollen grains removed in the periods of nocturnal visitors (29.4%;
530 $13,328 \pm 6,977$) and crepuscular bees at dusk (22.6%; $10,245 \pm 7,134$) and dawn (19.4%,
531 $8,831 \pm 8,733$), as well as the overlap of crepuscular and diurnal bees (21.2%, $9,610 \pm$
532 $4,351$) did not differ statistically (Figure 4D). However, the number of pollen grains
533 collected exclusively by diurnal bees (3.9%; $1,805 \pm 1,982$) was significantly lower when
534 compared to that removed by other floral visitors (Figure 4D).

535 At the end of the flower visits ($\sim 7:00$ h), an average of 160 (± 120 ; $n = 15$) pollen
536 grains were deposited on the stigmas, which is only 0.001% of the average number of
537 pollen grains produced per flower and corresponds to 1.03 pollen grains deposited on the
538 stigma per ovule.

539 In both situations, when visiting the flowers only at dawn (699 pollen
540 grains/min/anther) or in the two twilight periods (512 and 441 pollen grains/min/anther
541 at dusk and dawn, respectively), crepuscular bees removed pollen at the greatest rate from
542 a flower of *Pseudobombax longiflorum*. During the period in which crepuscular and
543 diurnal bees overlapped, pollen removal per minute per anther was 426 and 320 grains
544 (flowers visited only at dawn or in both twilights, respectively). The mean number of
545 pollen grains removed from the flowers of *Pseudobombax longiflorum* per minute was
546 minuscule during the long period when the flowers were accessible to nocturnal visitors
547 and diurnal. When crepuscular bees visited the flowers only at dawn and at dusks and
548 dawn, they removed respectively 30.4 and 51.5 times more than during pollen removal
549 by nocturnal visitor and 24.7 and 21.1 times more pollen grains than that of crepuscular
550 bees (Figure 5).

551 **4. DISCUSSION**

552 The study reveals that crepuscular bees gain most of the pollen of *P. longiflorum*
553 flowers. This efficient removal occurs mainly in the ~20-min twilight periods, when
554 crepuscular bees forage alone, without nocturnal and diurnal competitors. This confirms
555 the hypothesis of Wcislo et al. (2004) that it is strongly advantageous for crepuscular
556 bees, in terms of pollen gain, to conquer the short time-space without competitors. Their
557 ability to fly under dim-light conditions allows them to search for flowers during the
558 period of flower opening at dusk and before the flight activity of diurnal bees at dawn. In
559 the absence of crepuscular bees, the major pollen amount of *P. longiflorum* would go to
560 the introduced honeybees, their most abundant competitors in the early morning.
561 Astonishingly, most part of the pollen resources of this bat-pollinated tree species flows
562 to bees that do not contribute to its fruit set.

563 **4.1 Efficient pollen gain in the short twilight periods**

564 In the short twilight periods, when crepuscular bees foraged without competitors,
565 they collected > 40% of the pollen content of a flower of *P. longiflorum* in visits at dusk
566 and dawn and > 30% of the pollen in flowers available to these bees only at dawn.
567 Averaging both periods of exclusive crepuscular bee access (dusk and dawn), these bees
568 collect ~550 pollen grains per anther and minute when there are no further visitor groups.
569 This value of pollen removal is much higher than that removed by other visitors. This
570 high pollen collection efficiency of crepuscular bees is a consequence not only of their
571 individual pollen removal ability, but mainly because they visit the flowers when these
572 are still rich in pollen.

573 We do not know how many pollen grains are collected by crepuscular and how
574 many by diurnal bees in the period of foraging overlap of both bee groups between 5:50
575 h and 6:20 h. Considering, however, that the much larger females of crepuscular
576 *Ptiloglossa* grasp several anthers at once and remove pollen grains from their anthers
577 through sonication, similar to the less abundant carpenter bees, they might remove much
578 more pollen grains per visit than workers of honeybee and stingless bees, which collect
579 pollen just from individual anthers and much slower. Thus, a major part of pollen
580 removed during the period of overlap of diurnal and crepuscular bees might also flow to
581 the crepuscular bees.

582 The measured overall high pollen gain (> 50%) of crepuscular *Ptiloglossa* from
583 bat-pollinated *P. longiflorum* approximates that of narrow bee-plant relationships such as
584 those of oligolectic bee species and their specific host plants (Schlindwein et al. 2005;
585 Pick et al., 2011; Carvalho & Schlindwein, 2011; Cerceau et al., 2019, Siriani-Oliveira et
586 al., 2018), albeit with the difference that bees in the studied bat-pollinated species do not
587 contribute to the pollination of their hosts.

588 Pollen collection at dusk in fresh flowers of *Pseudobombax longiflorum* that still
589 contain the full pollen amount is remarkably relevant for the crepuscular *Ptiloglossa* bees.
590 Because there are no more flower visits of crepuscular bees to the fresh pollen-rich
591 flowers after 18:00 h, we assume that it is too dark for them to encounter flowers or return
592 to their nest again on the foraging trip. Thus, within the period of flower opening of *P.*
593 *longiflorum*, only less than one third of the flowers open early enough to be available for
594 these bees. Although crepuscular bees have a visual system adapted to foraging in dim-
595 light conditions (Warrant et al., 2004; Greiner et al., 2004, Greiner et al., 2005), the
596 number of light photons in the environment after 18:00 h is too low for these bees and
597 close to physiological noise in the photoreceptors, which decreases the confidence of the
598 visual signal (Warrant, 2017) in this period. Thus, light intensity limits the time of
599 foraging activity in crepuscular bees (Kelber et al., 2006, Liporoni et al., 2020).

600 In the short period of foraging at dusk, crepuscular bees gather 1.3 times the
601 amount of pollen collected per minute when compared to dawn. Flower visits of
602 crepuscular bees at dusk have also further impacts on the general pollen flow: the
603 nocturnal bat pollinators, which also feed on pollen of *Pseudobombax* (Gribel & Gibbs,
604 2002), encounter flowers with 22.6% less pollen per flower after crepuscular bee visits.
605 Moreover, our data reveal that the number of pollen grains shared with diurnal bee
606 competitors is reduced by 7% in flowers that were visited by crepuscular at dusk.
607 Therefore, flower visits at dusk (i) increase the total amount of pollen collected by
608 crepuscular bees, (ii) reduce the pollen flow to nocturnal visitors, and (iii) diminish the
609 pollen amount collected by diurnal bee competitors.

610 Curiously, bees of *Ptiloglossa* and carpenter bees inspect floral buds at dusk
611 before floral opening. Similar inspection flights have been observed for *Ptiloglossa*
612 *arizonensis* at buds of *Solanum elaeagnifolium* Cav., but at dawn (Linsley & Cazier,
613 1970; Shelly et al., 1993). This indicates that energy invest to locate floral buds or pre-

614 anthesis flowers in tree crowns is important for crepuscular bees and may be associated
615 with the strategy to encounter pollen-rich fresh or non-visited flowers.

616 In *P. longiflorum*, floral buds are dark brown and have a low light reflectance,
617 similar to those of the crepuscular bee-pollinated *Machaerium opacum* Vogel (Siqueira
618 et al. 2018). Bees of *Ptiloglossa* might thus be attracted mainly by olfactory floral cues
619 since the emission of strong floral scent is characteristic for these flowers (Carvalho et
620 al., 2012; Cordeiro et al., 2017, 2019; Krug et al., 2018, Siqueira et al. 2018). In this
621 context, it would be interesting to know whether crepuscular bees use also scents
622 unpleasant to humans, such as those that are typical of bat-pollinated flowers, to locate
623 floral resources. Further studies should investigate the role of olfactory and visual
624 memories in daily bud inspection and the location of newly opened flowers.

625 **4.2 Competition between crepuscular and diurnal bees**

626 Honeybees are by far the strongest competitors of crepuscular bees for the floral
627 resources of *P. longiflorum*. The carpenter bees, which arrive at the flowers a few minutes
628 before the honeybees, were only rare flower visitors. From ~5:50 h onward, honeybees
629 visited the flowers. Within 10 min, they were massively abundant, and often, we noted
630 more than 15 workers at a single flower searching for pollen and, albeit at less numbers,
631 for nectar. Similar cases have been reported for flowers of other species visited by
632 crepuscular bees and honeybees (Carneiro & Martins, 2012; Cordeiro et al., 2017,
633 Siqueira et al. 2018). Massive visits of honeybees occur due to their ability of efficient
634 communication and recruitment (von Fisch, 1967; Seeley, 1995; Dyer, 2002; Seeley,
635 2012). They are also strong competitors of the common native stingless bees (Wilms et
636 al., 1996). Their ability to fly earlier than native stingless bees and at low light intensities,
637 partly overlapping with the foraging period of crepuscular bees, probably makes them
638 such strong competitors. Even if pollen collection of individual honeybees is less efficient
639 because they collect pollen only from single anthers, they are extraordinarily abundant
640 from 6:00 h onward. In the absence of crepuscular bees, honeybees most likely would
641 obtain the entire available pollen supply of *P. longiflorum* after the nocturnal visits of
642 bats. On the other side, in the absence of the introduced honeybees, the crepuscular bees
643 would gain by far most of the pollen grains during the period of flight overlap with diurnal
644 bees.

645 **4.3 Implications of resource collection by bees for bat-pollinated *Pseudobombax***
646 ***longiflorum***

647 The floral opening time of *Pseudobombax longiflorum* is adjusted to the flight
648 period of bats, as in other chiropterophilous species (Vogel, 1954; van der Pijl, 1961;
649 Dobat & Peikert-Holle, 1985). Some of the flowers of the studied species, however, open
650 at dusk, when females of *Ptiloglossa* may remove about three million pollen grains from
651 one flower. If floral opening would occur earlier, crepuscular bees and even diurnal bees
652 could clean the anthers, and the flowers would become little attractive to bats. This would
653 have a strong negative impact on this species because bees are not effective pollinators
654 of *Pseudobombax longiflorum* due to size mismatch, a characteristic that seems to be true
655 also for other bat-pollinated species (Araujo et al., 2020). Therefore, the flower opening
656 time adjusted to the flight activity of bats should be influenced by the negative impact of
657 these efficient pollen-collecting non-pollinating bees. Flowers of the chiropterophilous
658 tree *Caryocar Brasiliense* Cambess, which are relevant pollen donors for crepuscular
659 bees, also set no fruits after intense flower visits of such bees (Araujo et al., 2020). This
660 seems to be the case also for other bat-pollinated species, especially those that provide
661 high amounts of pollen (Hernández-Montero & Sosa, 2016; Lassen et al., 2012; Sazima
662 & Sazima, 1978).

663 Due to the large amounts of pollen and nectar in bat-pollinated flowers (van der
664 Pijl, 1961; Vogel, 1968) and the great pollen gain of *Ptiloglossa* bees, shown here, we
665 suggest that crepuscular bees need to visit only few of these flowers to gather their larval
666 food supply. This causes less energy expenditure during their short-time dim-light
667 foraging. The predictability and quantity of pollen make these bat-pollinated trees
668 excellent reliable food sources for crepuscular bees. Nevertheless, it would be interesting
669 to obtain information on the pollination effectiveness of crepuscular bees on the flowers
670 of other bat-pollinated species and the quantity of floral resource removal in these flowers
671 by crepuscular bees to know whether the results obtained here may be generalized.

672 Although our study clearly demonstrates the advantages for crepuscular bees to
673 efficiently collect floral resources in a competitor-free space in the short dim-light periods
674 at dusk and dawn due to their ability to fly under low light conditions, a recent work
675 focusing on the paleotropical nocturnal carpenter bee *Xylocopa tranquebarica* (Fabricius)
676 reveals contrary results. These nocturnal carpenter bees mainly forage on residual
677 resources of diurnal flowers and strongly suffer competition with diurnal bees. Their

678 opportunism makes them less efficient foragers (Somanathan et al., 2020). These bees
679 show prolonged flight activity, are capable to fly also in the dark night and thus are true
680 nocturnals (Somanathan et al., 2020). This differs to the neotropical crepuscular bees
681 *Ptiloglossa*, with a flight activity essentially restricted to dim-light conditions. During
682 their short activity peaks at dusk and dawn, they demonstrate behavioral specialization to
683 efficiently explore fresh pollen-rich flowers and adjust their foraging to flower opening
684 and periods without competitors.

685 The advantages of the specialized resource collection of the crepuscular bees
686 demonstrated for bat-pollinated *Pseudobombax* most likely also apply to melittophilous
687 species with flower opening before or at dawn, where crepuscular are the first to explore
688 the pollen-rich flowers, as indicated for species of *Solanum*, *Machaerium* (Fabaceae) and
689 several Myrtaceae (Linsley & Cazier, 1970; Shelly et al., 1993; Cordeiro et al., 2017;
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691

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916 **FIGURES AND TABLES**

917 **TABLE 1** Flower-visiting bees of *Pseudobombax longiflorum* during the flowering
 918 seasons of 2018, 2019, and 2020 in the Serra do Cipó, Brazil. P = pollen; N = nectar.

Taxon	Sex	Habit	Resource collected P/N
Apidae			
<i>Apis mellifera</i> Linnaeus, 1758	♀	Diurnal	P/N
<i>Xylocopa (Neoxylocopa) grisescens</i> Lepeletier, 1841	♀	Diurnal	P/N
<i>Trigona hyalinata</i> (Lepeletier, 1836)	♀	Diurnal	P/N
Colletidae			
<i>Ptiloglossa xanthotricha</i> Moure, 1945	♀/♂	Crepuscular	P/N
<i>Ptiloglossa stafuzzai</i> Moure, 1945	♀/♂	Crepuscular	P/N

919

920 **TABLE 2** Fruit set after natural pollination (unbagged flowers) and flowers accessible at
 921 dusk (until 18:00 h) and dawn onward (after 05:20 h), periods of bee flower visits.
 922 Flowers were bagged during the night. Different letters represent significant differences
 923 between relative frequencies, $\chi^2 = 14.1$, $n = 32$, $p < 0.001$.

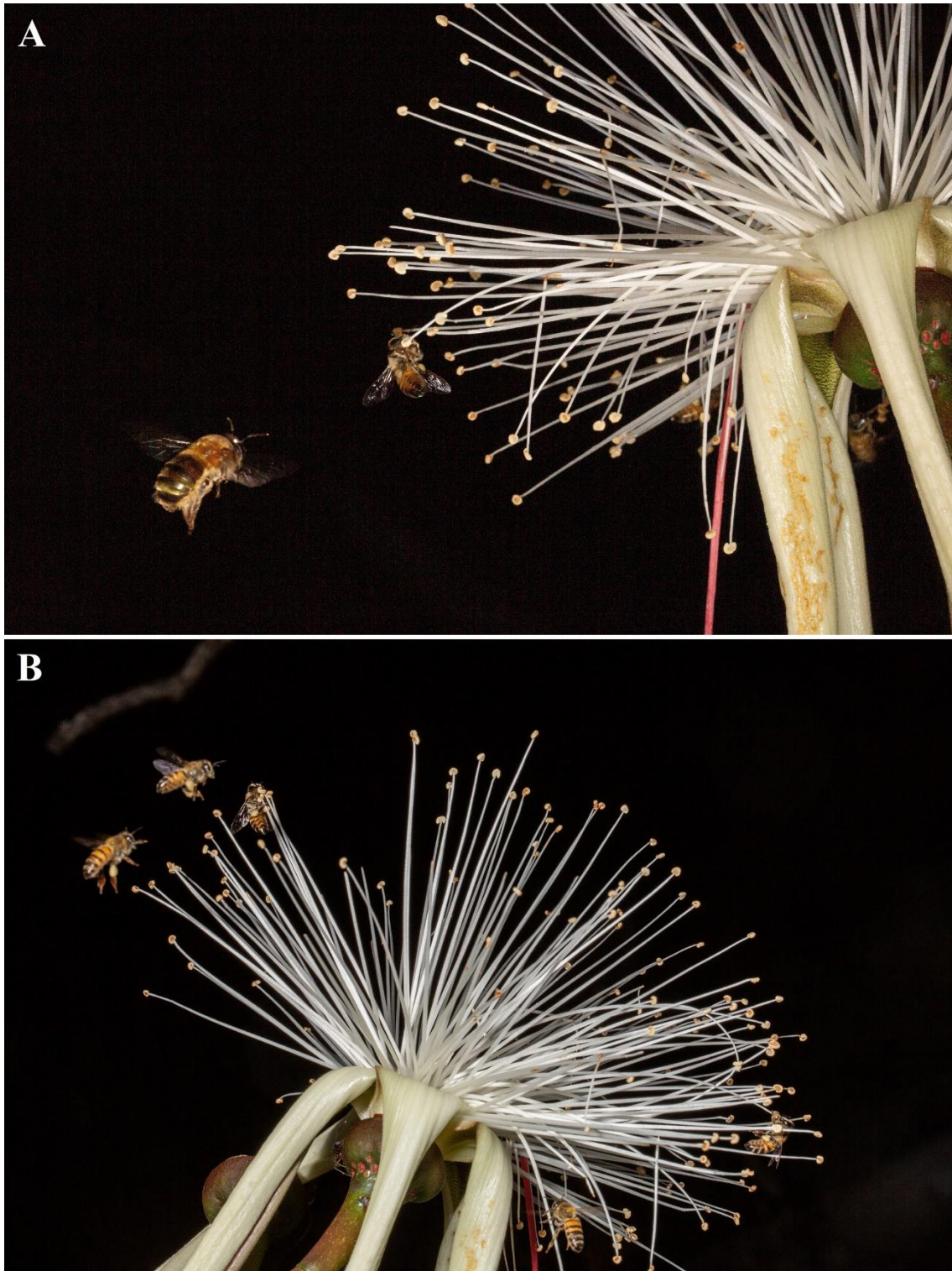
Treatment	N	Fruit set (%)	Seed set (n, SD)
Natural pollination (unbagged flowers)	32	14 (43%) ^a	61,8 ±19
Bee pollination (flowers bagged during the night)	32	1 (3,1%) ^b	30

924



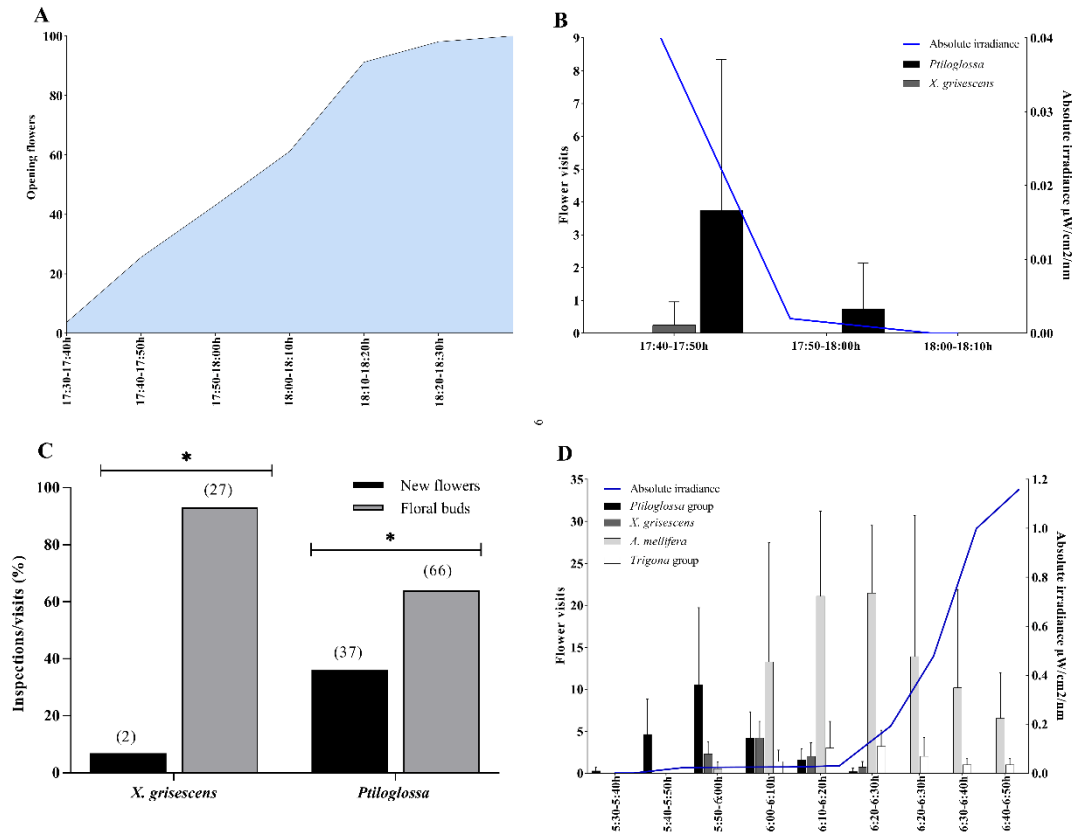
925

926 **Figure 1.** New flower of *Pseudobombax longiflorum* at dusk. The large brush blossoms
927 open between 17:30 h and 18:30 h, exposing the showy white filaments and anthers. The
928 red style with the inconspicuous stigma overtops the stamens by ~2.5 cm.



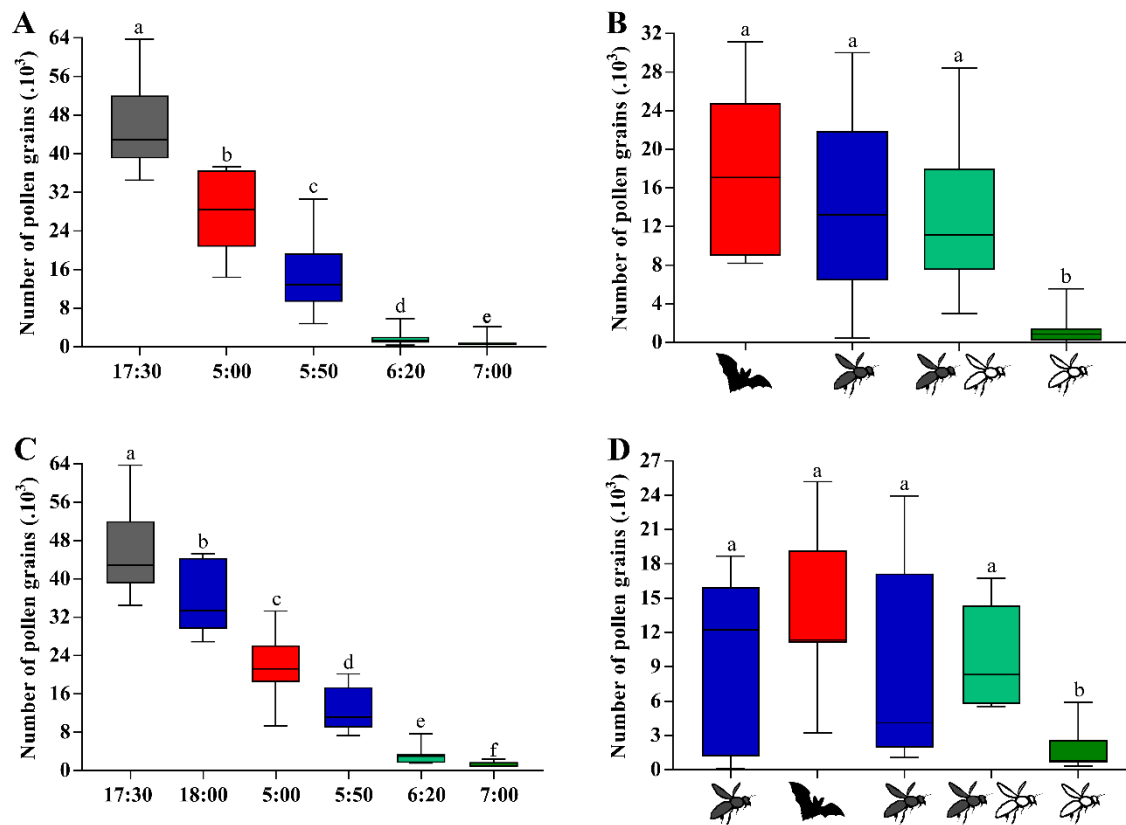
929

930 **Figure 2.** (a) A female of *Ptiloglossa* approaching a flower of *Pseudobombax longiflorum*
931 at dawn and a honeybee worker (*Apis mellifera*) collecting pollen from a single anther.
932 (b) Several honeybees at a flower during peak-visiting after dawn.



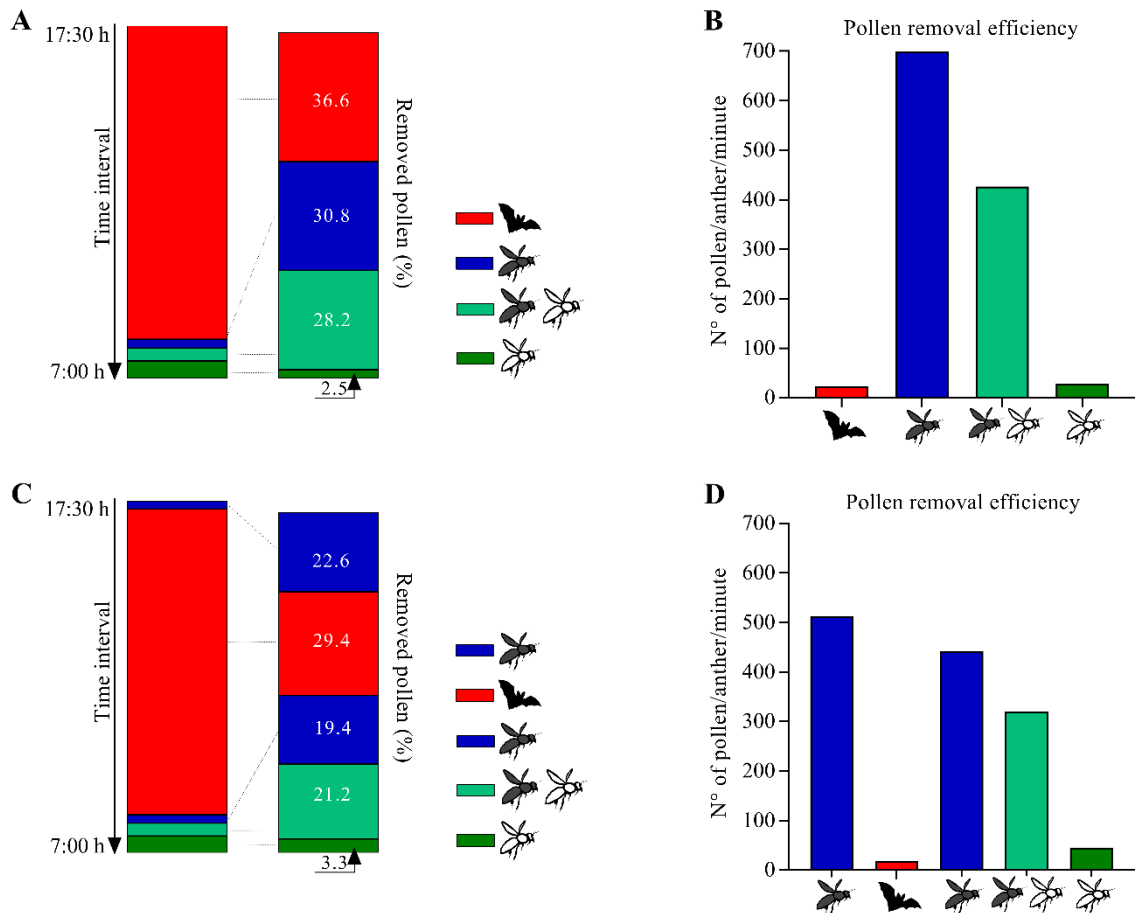
933

934 **Figure 3.** (a) Cumulative curve of floral openings of *Pseudobombax longiflorum* at dusk
 935 (n = 160 flowers), in 10-min intervals. (b) Floral visits in 10-min intervals at dusk (mean
 936 \pm SD). The blue line indicates light intensities. (c) Percentage of floral bud inspections
 937 and visits to new flowers at dusk, respectively, by bees of *Ptiloglossa* and *Xylocopa*
 938 *grisescens*. Number in parentheses represents the number of inspections/visits.
 939 * Significant differences between the number of inspections and floral visits. (d) floral
 940 visits in 10-min intervals at dawn and in the early morning hours (mean \pm SD). Visits of
 941 honeybees started at ~6:00 h. The blue line indicates the light intensities.



942

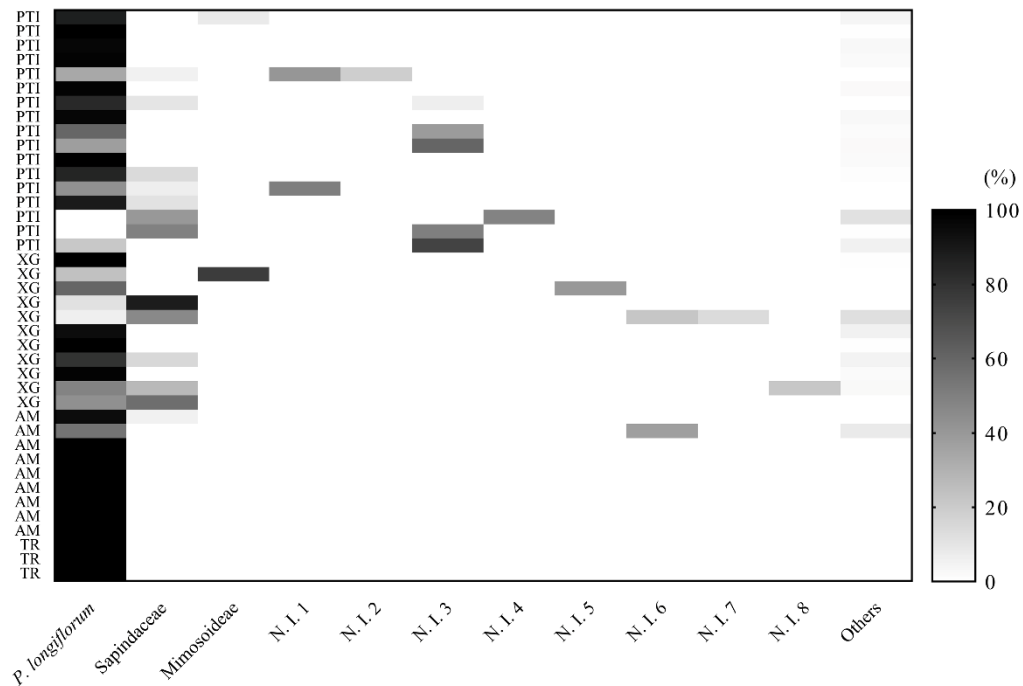
943 **Figure 4.** Pollen grains of *Pseudobombax longiflorum* per anther throughout anthesis.
 944 The time intervals are related to the phases of daytime and activity periods of the different
 945 floral visitor groups. **(a, c)** Number of pollen grains per anther. **(a)** Crepuscular bee visits
 946 only at dawn; **(c)** crepuscular bee visits at dusk and dawn. Key: grey bar, beginning of
 947 anthesis at dusk; blue bars, twilight; red bar, night; light green bar, early morning; green
 948 bars, day. Different letters indicate significant differences ($P < 0.05$) by planned
 949 comparison. **(b, d)** Pollen fate per period, **(b)** in flowers visited by crepuscular bees at
 950 dusk and dawn and **(d)** only at dawn. Both graphs represent the number of pollen grains
 951 removed by floral visitor groups. Key: black bat, nocturnal visitors; grey bee, crepuscular
 952 bees; grey and white bees, overlap of crepuscular and diurnal bees; white bee, diurnal
 953 bees. Different letters indicate significant differences ($p < 0.05$) by planned comparison.
 954 Box plots indicate the median (solid line) and dispersal (lower and upper quartiles, and
 955 outliers) of the number of pollen grains per floral visitor.



956

957 **Figure 5.** Pollen removed by different floral visitors per time interval throughout anthesis
 958 of *P. longiflorum* flowers. **(a, c)** Time interval that each floral visitor had to collect pollen
 959 (in the left) and the amount of pollen removed (%) by each floral visitor (in the right) at
 960 dawn (a and c) and dusk (c). Black bat = nocturnal visitors; grey bee = crepuscular bees;
 961 grey and white bee = overlap of crepuscular and diurnal bees; white bee = diurnal bees.
 962 **(b, d)** Pollen removal efficiency per anther in 1 minute by floral visitors at dawn (c and
 963 d) and dusk (d). Black bat = nocturnal visitors; grey bee = crepuscular bees; grey and
 964 white bee = overlap of crepuscular and diurnal bees; white bee = diurnal bees.

965 SUPPORTING INFORMATION



966

967 **Figure S1.** Relative frequencies of pollen types found in the pollen loads of crepuscular
 968 and diurnal bees collected in *Pseudobombax longiflorum* flowers. The heatmap
 969 representation shows the percentages of each pollen type (columns) on each female
 970 (rows). PTI = *Ptiloglossa*. XG = *Xylocopa grisescens*. AM = *Apis mellifera*. TH =
 971 *Trigona hyalinata*. N. I. = pollen morphotypes not identified.

CAPÍTULO II**972**
973 PISTAS FLORAIS VISUAIS E OLFATIVAS UTILIZADAS NO CREPÚSCULO
974 POR ABELHAS EM BUSCA DE FLORES QUIROPTERÓFILAS
975

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991 **RESUMO**

992 As interações entre abelhas e flores melitófilas são mediadas principalmente por pistas
993 olfativas e visuais. As abelhas crepusculares, que buscam recursos florais somente sob
994 baixa luz, são conhecidas por usar pistas olfativas para encontrar flores. No entanto,
995 embora essas abelhas tenham um sistema visual adaptado para voar em condições de
996 pouca luz, o papel de pistas visuais para localizar flores não é conhecido nestas abelhas.
997 Além de flores melitófilas, abelhas podem coletar pólen e néctar em flores quiropterófilas.
998 Essas flores, no geral, possuem cores claras e um forte desagradável odor floral. Contudo,
999 não se sabe se as abelhas usam este cheiro para encontrar as flores sob a baixa luz do
1000 crepúsculo. Perguntamos: Quais traços florais as abelhas usam como pistas para encontrar
1001 as flores quiropterófilas sob baixa luz? Qual o papel destas pistas durante o forrageamento
1002 das abelhas? Para responder essas perguntas escolhemos como modelo as flores de
1003 *Pseudobombax longiflorum* (Malvaceae), uma típica espécie quiropterófila com flores
1004 brancas e forte odor floral. As flores são visitadas por abelhas *Ptiloglossa* (Colletidae) no
1005 crepúsculo e início da manhã. As visitas dessas abelhas sobrepõem com as da abelha
1006 diurna *Xylocopa grisescens* (Apidae). Nós analisamos a refletância espectral e os
1007 compostos voláteis das flores de *P. longiflorum* e fizemos dois bioensaios: Testamos se
1008 as *Ptiloglossa* usavam os odores e as cores de *P. longiflorum*, isolados e combinados,
1009 como pista floral. Em seguida, produzimos flores artificiais com a cor dos filetes e odores
1010 de *P. longiflorum* isolados e combinados. Testamos novamente se estas características
1011 florais eram usadas como pista pelas abelhas *Ptiloglossa* e *X. grisescens*. O número de
1012 respostas totais (pouso + aproximação) de abelhas crepusculares nos dois experimentos
1013 foram maiores em flores com os odores e cores combinados do que em flores com apenas
1014 uma dessas características. *Xylocopa grisescens* responderam igual as flores com apenas
1015 a cor floral e cor e odores florais combinados, e em menor frequência a flores com apenas
1016 os odores. Nossos resultados indicam que as abelhas usam os odores e cores das flores de
1017 *P. longiflorum* como pista para as localizar durante o amanhecer. Para ambas as espécies
1018 estudadas os odores florais de *P. longiflorum* desencadeavam apenas aproximação. Os
1019 pousos ocorriam apenas quando a pista visual estava presente. Além disso, para as abelhas
1020 crepusculares odores e cores são igualmente importantes na busca por flores de *P.*
1021 *longiflorum*. Enquanto *X. grisescens* usa preferencialmente a cor como pista para localizar
1022 as flores.

- 1023 **PALAVRAS-CHAVE:** abelhas crepusculares, abelhas noturnas, *Pseudobombax*
1024 *longiflorum*, *Ptiloglossa*, *Xylocopa*, compostos florais, refletância flora, pista floral.

1025 1. INTRODUÇÃO

1026 As interações entre as abelhas e as flores são mediadas por pistas florais, como as
1027 cores, odores, forma e simetria (Faegri e van der Pijl, 1979; Giurfa e Lehrer, 2001; van
1028 der Kooi et al., 2016; van der Kooi et al., 2019; Barragán-Fonseca et al., 2019). Dentre as
1029 diferentes pistas florais usadas pelas abelhas para localizar e reconhecer as flores,
1030 destacam-se cor e odor, que podem ser usados isolados ou combinados (Giurfa et al.,
1031 1994; Srinivasan et al., 1998, Kunze e Gumbert, 2001; Chittka e Raine, 2006; Milet-
1032 Pinheiro et al., 2012; Yan et al., 2016; Dötterl e Vereecken 2010; Lawson et al., 2018;
1033 Rachersberg et al., 2019; Koethe et al., 2020).

1034 Durante o forrageamento, pistas visuais são usadas pelas abelhas para detectar e
1035 discriminar flores com diferentes quantidades de recursos florais (Wertlen et al., 2008).
1036 No geral, as cores são percebidas pelas abelhas quando estão próximas das flores, pois a
1037 resolução espacial dos seus olhos compostos não permite a detecção destas pistas visuais
1038 a distâncias maiores (Giurfa et al., 1996; Chittka e Raine, 2006). Porém, quando flores
1039 ocorrerem em grande densidade, abelhas podem as localizar em distâncias maiores
1040 (Giurfa et al., 1996). Já pistas olfativas podem atrair e guiar abelhas às flores em curtas e
1041 longas distâncias (Dötterl e Vereecken, 2010). Em sistemas de polinização noturna, as
1042 pistas olfativas são especialmente importantes para guiar os besouros, mariposas e
1043 morcegos até as flores. A coleta dos recursos florais é desencadeada pela combinação das
1044 pistas olfativas e visuais (Gottsberger e Gottsberger, 1991; von Helversen et al., 2000;
1045 Majetic et al., 2007; Balkenius et al., 2006; Klahre et al., 2011).

1046 Diferentemente de abelhas diurnas que buscam por recursos florais durante o dia,
1047 abelhas crepusculares buscam flores apenas durante o crepúsculo (Warrant et al., 2004;
1048 Kelber et al., 2006), período em que o nível de luz no ambiente é baixo (Theobald et al.,
1049 2007; O'Carroll e Warrant, 2017). Para melhorar a confiabilidade visual durante o
1050 crepúsculo, os olhos das abelhas crepusculares possuem adaptações que aumentam a
1051 sensibilidade à luz (Warrant et al., 2004; Greiner et al., 2004 ab; Frederiksen et al., 2008).
1052 Abelhas crepusculares da espécie *Megalopta genalis* Meade-Waldo, 1916, usam as
1053 variações no padrão formado pelo dossel das árvores como pistas visuais para navegar
1054 (Chaib et al., 2021), enquanto *Xylocopa tranquebarica* Fabricius, 1804, abelha noturna,
1055 reconhece cores durante a noite (Somanathan et al., 2008). Ambas as espécies usam
1056 marcas visuais ao redor do ninho para os identificar (Warrant et al., 2004; Somanathan et

1057 al., 2008). Contudo, ainda não foi demonstrado se abelhas crepusculares usam pistas
1058 visuais para reconhecer flores.

1059 As pistas olfativas são importantes para atrair abelhas crepusculares para flores
1060 melitófilas (Cordeiro et al., 2017; Krug et al., 2018; Siqueira et al., 2018; Cordeiro 2019;
1061 Martinez-Martinez et al., 2021). Alguns estudos demonstraram que abelhas crepusculares
1062 são atraídas por voláteis florais, e assim essa pista exerce um papel importante na busca
1063 por flores pelas abelhas durante o crepúsculo (Knoll e Santos, 2012; Carvalho et al., 2012,
1064 Cordeiro et al., 2017; Krug et al., 2018). Recentemente foi descrito que abelhas
1065 crepusculares inspecionam botões florais de uma espécie quiropterófila, *Pseudobombax*
1066 *longiflorum* (Mart. et Zucc.) A. Robyns (Araújo et al., 2021), mas não foi demonstrado se
1067 essas abelhas usam esses odores como pista.

1068 Abelhas crepusculares visitam não apenas flores melitófilas, mas também flores
1069 adaptadas a polinização por morcegos (Roulston, 1997; Wcislo et al., 2004; Araujo et al.,
1070 2020, Araújo et al., 2021). Flores quiropterófilas, no geral, possuem cores claras e forte
1071 odor floral (Vogel, 1954; van der Pijl, 1961; Faegri and van der Pijl, 1979). Dentre os
1072 voláteis produzidos por essas flores, destacam-se compostos de enxofre, que
1073 frequentemente são responsáveis pelo odor desagradável para o nariz humano (Bestmann
1074 et al., 1997; von Helversen et al., 2000). Recentemente, foi demonstrado que as flores de
1075 *Caryocar brasiliense* Cambess., espécie quiropterófila visitada também por abelhas
1076 diurnas e crepusculares (Araujo et al., 2020), produzem majoritariamente derivados de
1077 ácidos graxos além de voláteis contendo enxofre (Paiva et al., 2019). Contudo, não se
1078 sabe se as abelhas usam esses odores para encontrar as flores quiropterófilas.

1079 *Pseudobombax longiflorum* é uma típica espécie quiropterófila cujas flores são
1080 visitadas no crepúsculo pelas abelhas crepusculares *Ptiloglossa stafuzzai* Moure, 1945 e
1081 *P. xanthotricha* Moure, 1945, e pela espécie diurna *Xylocopa grisescens* Lepeletier, 1841,
1082 que também visita as flores em horários com baixa luminosidade (Araújo et al., 2021).
1083 Ao anoitecer, independentemente da presença de flores novas, *Ptiloglossa* e *X. grisescens*
1084 frequentemente voam ao redor das copas das árvores de *P. longiflorum* e aproximam-se
1085 de botões florais (Araújo et al., 2021). Nesses voos, raramente *X. grisescens* visitam flores
1086 novas, enquanto 36% dos voos das fêmeas das abelhas *Ptiloglossa* coincidem com a
1087 abertura floral (Araújo et al., 2021). Ao amanhecer, as flores são visitadas no crepúsculo
1088 por abelhas *Ptiloglossa*, e quando o dia começa a amanhecer, *X. grisescens* se juntam a

1089 elas nas flores (Araújo et al., 2021). Contudo, não se sabe quais características forais de
1090 *P. longiflorum* são usadas como pista por essas abelhas sob baixa luz.

1091 O objetivo desse estudo foi responder as seguintes questões: Quais características
1092 florais as abelhas usam como pistas para encontrar as flores de *P. longiflorum* sob baixa
1093 luz? Qual o papel destas pistas durante o forrageamento das abelhas? Para isso
1094 descrevemos a refletância espectral e os compostos voláteis das flores de *P. longiflorum*.
1095 Testamos se as abelhas *Ptiloglossa* usam o odor e a cor, combinados ou isolados, das
1096 flores de *P. longiflorum* como pista. Em seguida, para entender melhor quais possíveis
1097 estímulos eram usados pelas abelhas *Ptiloglossa* e *X. grisescens* para localizar essas
1098 flores, fizemos um segundo teste com flores artificiais. A parte mais visível das flores de
1099 *P. longiflorum* são os filetes em forma de pincel. Assim, produzimos flores artificiais com
1100 o mesmo padrão dessa estrutura. Também produzimos flores artificiais pretas com extrato
1101 floral de *P. longiflorum*, excluindo a possibilidade de as abelhas terem acompanhado o
1102 crescimento do botão floral e memoriado a posição de flores novas (Araújo et al., 2021).
1103 Testamos se as abelhas usam esses estímulos, combinados ou isolados, para encontrar as
1104 flores artificiais.

1105

1106 **2. MATERIAIS E MÉTODOS**

1107 **2.1 Área de estudo**

1108 O estudo foi realizado em junho de 2019, no período de floração de *P.*
1109 *longiflorum*, em uma área de Cerrado sobre afloramento calcário em Santana do Riacho
1110 na Serra do Cipó, Minas Gerais, Brasil (19°18'40,7 "S 43°36'42,2"W; 953 m).

1111 **2.2 Espécies estudadas**

1112 *Pseudobombax longiflorum* é uma árvore decídua com até 25 m de altura. A
1113 espécie ocorre em quase todo o território brasileiro (Botanical Information and Ecology
1114 Network - BIEN). Suas flores em forma de pincel são brancas e produzem durante toda a
1115 antese um forte odor floral. A antese inicia entre 17:30 - 18:30 h e a senescência ocorre
1116 por volta das 16:00 h do dia seguinte. As flores possuem em média ~290 estames, com
1117 12,3 cm de comprimentos, que abrem no início da antese e produzem cerca de 13.500.000
1118 grãos de pólen (Araújo et al., 2021).

1119 Na área de estudo as flores são visitadas pelas abelhas crepusculares *Ptiloglossa*
1120 *stafuzzai* e *Ptiloglossa xanthotricha* no crepúsculo matutino e vespertino (Figura 1A), e
1121 no início da manhã pelas abelhas carpinteiras *Xylocopa grisescens* (Figura 1B), *Apis*
1122 *mellifera* Linnaeus, 1758 e *Trigona hyalinata* (Lepeletier, 1836). Ao amanhecer
1123 *Ptiloglossa* visitam as flores entre 5:30 - 6:20 h, período em que a intensidade de luz no
1124 ambiente está entre $1,98 \times 10^{-4}$ e $0,19 \mu\text{W}/\text{cm}^2$. As abelhas carpinteiras visitam as flores
1125 entre 5:50 - 6:20 h, período em que a intensidade luminosa está entre $2,5 \times 10^{-2}$ e $0,19$
1126 $\mu\text{W}/\text{cm}^2$. As abelhas não polinizam as flores de *P. longiflorum*, mas coletam cerca de
1127 60% do conteúdo polínico (Araújo et al., 2021).

1128 **2.3 Perfume floral, amostragem e análise**

1129 Flores de seis árvores de *P. longiflorum* foram removidas após a abertura floral ~
1130 18:30 h e colocadas individualmente em sacos inodoros de poliéster (40 cm, Wyda). Os
1131 extratos dos perfumes florais para os bioensaios foram coletados utilizando o método
1132 *headspace* dinâmico em 15 flores. O ar enriquecido com voláteis foi sugado através de
1133 tubos adsorventes por 2 horas usando uma bomba de vácuo (G12 / 01 EB; Rietschle
1134 Thomas, Puchheim, Alemanha) com fluxo de ar constante ajustado por fluxômetro para
1135 200 ml/min. Os tubos adsorventes foram feitos de vidro de quartzo contendo Tenax-TA
1136 60–80 e 1 Carbotrap B 20–40 (ambos Supelco, Bellefonte, EUA) fixados com lã de vidro.
1137 Cada tubo adsorvente foi eluído com 0,4 ml de acetona pura (grau HPLC $\geq 99,9\%$,
1138 SIGMA). Os extratos resultantes foram armazenados a $\sim -4^\circ\text{C}$ e utilizados no bioensaio
1139 com flores artificiais.

1140 Para identificar e quantificar os compostos florais, coletamos os odores florais por
1141 microextração em fase sólida (SPME) de 10 flores (4 plantas). Uma fibra StableFlex
1142 (DVB / CAR / PDMS, 50 μm , Supelco, PA, EUA) foi exposta dentro dos sacos plásticos
1143 contendo uma flor por 1 hora. As fibras de SPME foram armazenadas a 0°C durante o
1144 transporte do campo para o laboratório analítico, aonde foram dessorvidas termicamente
1145 para análise por GC-MS.

1146 As análises de GC-MS foram realizadas em um cromatógrafo à gás Shimadzu
1147 GC2010 acoplado a um espectrômetro de massa Shimadzu QP2010 Plus (EI). As fibras
1148 de SPME foram expostas no modo de injeção splitless, a 270°C . Foi utilizada uma coluna
1149 capilar RTX-5 (Restek, PA, EUA; 30 m \times 0,25 μm \times 0,25 mm), com programação de
1150 gradiente de temperatura iniciando em 50°C mantida por 1 min e uma taxa de aumento

1151 de 7 ° C.min⁻¹ até 270 ° C. A determinação quantitativa dos componentes do extrato foi
1152 realizada empregando uma mistura de vários terpenóides de referência e derivados de
1153 ácidos graxos como padrão externo. Os índices de retenção (IR) foram calculados usando
1154 padrões comerciais de *n*-alcanos (C₁₀-C₂₆) como referência (parâmetros do equipamento
1155 conforme descrito acima). A elucidação estrutural foi realizada por comparação de dados
1156 em bibliotecas de espectros de massas e de índices de retenção (NIST, FFNSC2, Wiley).

1157 **2.4 Espectro de refletância das flores**

1158 A refletância espectral do estilete, filetes, anteras com pólen e a porção adaxial
1159 das pétalas das flores de *P. longiflorum* e flores artificiais foi medida com uma sonda de
1160 fibra óptica bifurcada (R400-7-UV-VIS, Ocean Optics) conectada a um
1161 espectrofotômetro (USB2000 + UV-VIS-ES, Ocean Optics) calibrado entre 300 e 700 nm
1162 usando-se uma fonte de luz de deutério / tungstênio (DH-2000-BAL, Ocean Optics). As
1163 medições foram feitas em um ângulo de 45° em relação à superfície que estava sendo
1164 medida. A distância entre a estrutura floral e o detector de luz foi ajustada em 0,5 cm
1165 usando um suporte de sonda (RPH-1, Ocean Optics). A iluminação na faixa do UV-VIS
1166 foi fornecida com uma fonte de luz de xenônio pulsado (PX-2, 220–750 nm, Ocean
1167 Optics).

1168 O modelo hexagonal de percepção cromática (Chittka, 1992) baseado nas curvas
1169 de absorção espectral dos fotorreceptores de himenópteros (Peitsch et al., 1992) foi
1170 utilizado para avaliar como os espectros refletidos pelo estilete, filetes, anteras e pétalas
1171 de *P. longiflorum*, e também pelas flores artificiais, são discriminados pelas abelhas. Cada
1172 *locus* de cor indica a posição angular e a distância perceptiva da estrutura floral em relação
1173 ao centro do hexágono, que representa a refletância do fundo (*background*). Os cantos do
1174 hexágono representam as faixas de luz absorvidas na visão tricromática da abelha (UV,
1175 azul e verde) e suas combinações (UV-azul, UV-verde e azul-verde). Quanto maior é a
1176 distância perceptual (ΔS) do centro, mais a cor da estrutura visual se contrasta ao fundo
1177 (Figura 2). Igualmente, quanto maior é ΔS entre duas cores no hexágono, maior é o
1178 contraste entre as mesmas. Estudo em abelhas melíferas mostram que a cromaticidade
1179 das estruturas é percebida como distinta para as abelhas quando $\Delta S \geq 0,11$ (Dyer et al.,
1180 2012). Quando $\Delta S > 0,04$ e $< 0,1$, os estímulos são dificilmente discriminados. Já
1181 estímulos visuais com $\Delta S \leq 0,04$ são indistinguíveis (Dyer et al., 2012).

1182 Visto que *P. longiflorum* perde sua folhagem durante a floração e suas flores ficam
1183 suspensas nos galhos, não foi utilizado como medida de fundo da cena visual o espectro
1184 de refletância da folhagem. No lugar, utilizamos um fundo acromático padrão ($q = 0.33$
1185 para receptores S, M e L; Chittka, 1992), uma vez que em o fundo da cena natural era
1186 geralmente composto pelo céu do crepúsculo, cujas propriedades espectrais variavam.

1187 **2.5 Bioensaios**

1188 Para determinar as pistas florais utilizadas pelas abelhas para localizar flores de
1189 *P. longiflorum*, foram realizados dois bioensaios: um com flores naturais e outro com
1190 flores artificiais. Os bioensaios foram conduzidos durante as visitas das abelhas no
1191 crepúsculo matutino e no início da manhã (05:30 - 06:20 h). Em ambos os experimentos,
1192 as flores foram dispostas a uma distância de 50 cm a 100 cm uma da outra. A posição das
1193 flores alterava de acordo com a disponibilidade das flores no dia do experimento.

1194 *Bioensaio com flores naturais manipuladas.* Estabelecemos um teste de escolha
1195 quádrupla com os seguintes tratamentos: (i) cor, (ii) odor, (iii) cor + odor, (iv) odor e cor
1196 ausente. Cada tratamento teve um total de 15 flores. Ao longo do período de floração nós
1197 acompanhamos o desenvolvimento dos botões florais em diferentes árvores. Dessa forma,
1198 conseguimos obter, ao longo dos 10 dias de experimento, uma média de ~2 plantas por
1199 dia com 4 flores novas cada. As abelhas tiveram as seguintes opções:

1200 (i) Cor: as flores foram ensacadas com sacos plásticos transparentes, para evitar a
1201 emissão de odores para a atmosfera. Os sacos foram firmemente amarrados nos ramos
1202 sem folhas, com um cordão. A base do saco foi fechada ao redor do galho com fita adesiva
1203 transparente. Sépalas, pétalas e filetes permaneciam facilmente visíveis (Figura 1C).

1204 (ii) Odor: as flores foram ensacadas com sacos plásticos pretos impermeáveis à
1205 luz, excluindo assim a visibilidade das flores. O saco plástico preto foi perfurado com
1206 pequenos orifícios em toda extensão (diâmetro ~ 1 mm), permitindo que o perfume floral
1207 exalasse para o ambiente externo (Figura 1D). O cheiro forte típico das flores de *P.*
1208 *longiflorum* era facilmente detectável pelo nariz humano nestas flores.

1209 (iii) Odor + cor: flores foram deixadas não ensacadas, livremente acessíveis aos
1210 visitantes florais.

1211 (iv) Odor e cor ausentes: as flores foram ensacadas em sacos transparentes e sacos
1212 plásticos pretos não perfurados.

1213 As respostas comportamentais das abelhas foram registradas como **1)**
1214 aproximações: voos com redução de velocidade em direção à flor, sem pouso ou **2)**
1215 pousos: aproximação seguida de pouso nas flores. O experimento foi conduzido entre 5 e
1216 15 de junho de 2019.

1217 *Bioensaio com flores artificiais.* O experimento com flores artificiais restringiu as
1218 escolhas das abelhas aos odores florais e cor dos filetes de *P. longiflorum*, excluindo
1219 outros traços florais como contraste entre as estruturas florais, e presença de pólen e
1220 néctar. Também, ao utilizar flores artificiais excluíamos a possibilidade de as abelhas
1221 encontrarem as flores novas por terem acompanhado o crescimento do botão floral, já que
1222 elas os inspecionavam diariamente (Araújo et al., 2021). Neste bioensaio, anotamos as
1223 respostas das abelhas *Ptiloglossa* e *Xylocopa grisescens*. Estabelecemos testes de
1224 quádrupla escolha como no experimento de flores naturais manipuladas: (i) cor, (ii) odor,
1225 (iii) odor + cor, (iv) odor e cor ausentes.

1226 As quatro flores artificiais de cada um dos tratamentos eram fixados em 2 plantas
1227 de *P. longiflorum*. O experimento foi repetido 15 vezes. Cada flor artificial foi
1228 posicionada na extremidade de um ramo. Todas as flores artificiais tinham formato
1229 semelhante das flores naturais. As abelhas tiveram as seguintes opções:

1230 **(i) Cor:** a parte mais visível das flores de *P. longiflorum* são os filetes. Assim,
1231 escolhemos um papel com refletância espectral semelhante de filetes (figura 2) para
1232 preparar as flores artificiais (Figura 1E). Não foi adicionado extrato de cheiro floral.

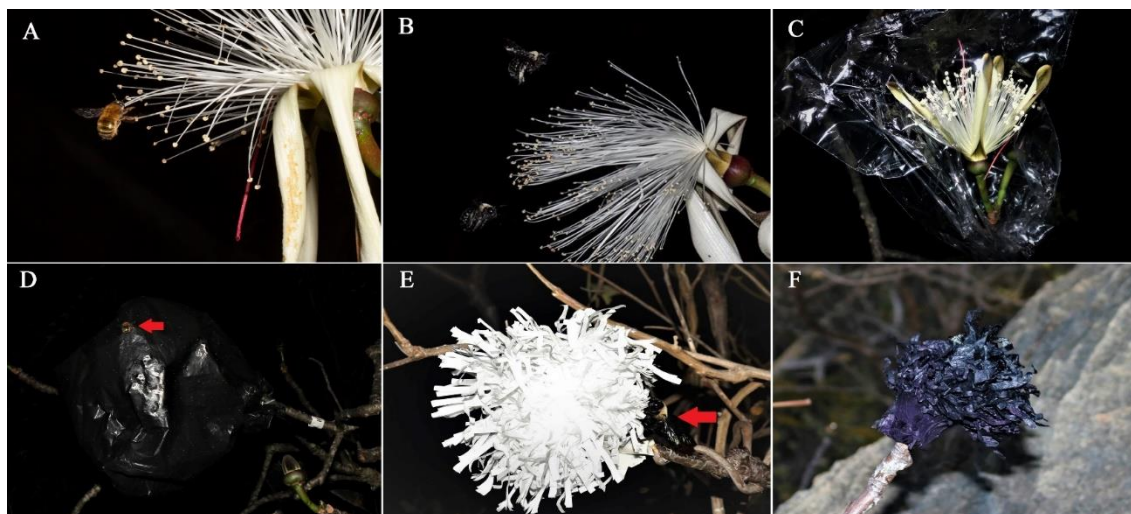
1233 **(ii) Odor:** para obter os extratos florais, coletamos os odores florais de flores novas
1234 de seis plantas de *P. longiflorum* usando método de *headspace* dinâmico (veja acima
1235 “Extratos de perfume floral, amostragem e análise”). Em cada flor artificial, adicionamos
1236 0,20 ml do extrato de cheiro floral. As flores artificiais foram feitas com papel preto
1237 (Figura 1F).

1238 **(iii) Odor + cor:** usamos flores artificiais brancas, ver (i), com 0,20 ml do extrato
1239 aromático.

1240 **(iv) Odor e cor ausentes:** usamos flores artificiais pretas sem extrato floral.

1241 As respostas comportamentais das abelhas foram registradas como **1)**
1242 aproximações: voos com redução de velocidade em direção à flor, sem pouso ou **2)**

1243 pousos: aproximação seguida de pouso nas flores. O experimento com flores artificiais
 1244 foi realizado entre 20 e 27 de junho de 2019.



1245

1246 **Figura 1.** Flores naturais e artificiais utilizadas nos bioensaios. A) uma fêmea de
 1247 *Ptiloglossa* visitando uma flor de *P. longiflorum*. B) Duas fêmeas de *Xylocopa grisescens*
 1248 voando em direção a uma flor de *P. longiflorum*. C) flor natural em um saco plástico
 1249 transparente evitando a emissão de odores para o ambiente. D) saco plástico preto com
 1250 pequenos orifícios cobrindo uma flor de *P. longiflorum*. Uma fêmea de *Ptiloglossa* (seta
 1251 vermelha) se aproximando ao saco preto perfurado com flor ensacada. E) Uma fêmea de
 1252 *Xylocopa grisescens* (seta vermelha) pousada em uma flor branca artificial. F) flor preta
 1253 artificial.

1254 2.6 Análise estatística

1255 *Bioensaio com flores naturais manipuladas.* Construímos um modelo linear geral (GLM)
 1256 com distribuição de Poisson, para averiguar se o número de respostas totais das abelhas
 1257 *Ptiloglossa* nas flores era influenciado pelas cores e odores florais, sozinhos ou
 1258 combinados. O número de respostas totais correspondia a soma dos pousos e das
 1259 aproximações. Usamos o número de respostas totais como variável dependente e as flores
 1260 manipuladas como variável preditora. Por fim, para averiguar se havia diferença entre o
 1261 número de respostas totais das abelhas entre as flores manipuladas (odor, cor, odor + cor),
 1262 realizamos a comparação planejada.

1263 Em flores com a cor isolada as abelhas *Ptiloglossa* pousavam e aproximavam.
 1264 Para avaliar se havia diferença entre esses dois comportamentos, construímos um modelo
 1265 linear generalizado misto (GLMM) com distribuição de Poisson. Usamos o número de

1266 respostas como variável dependente, as respostas comportamentais (pouso e
1267 aproximação) como variável preditora e a flor como variável aleatória.

1268 *Bioensaio com flores artificiais.* Construimos um GLMM com distribuição de Poisson
1269 para testar o efeito da cor dos estames e dos odores florais, combinados e isolados, no
1270 número de respostas totais das abelhas *Ptiloglossa* e *Xylocopa grisescens* às flores
1271 artificiais. Usamos o número de respostas totais das abelhas às flores como variável
1272 dependente. Espécies (*Ptiloglossa* e *X. grisescens*) e flores artificiais (odor, cor, odor +
1273 cor) como variáveis preditoras e a flor como variável aleatória. Por fim, para averiguar se
1274 havia diferença entre o número de respostas totais das abelhas entre as flores artificiais
1275 (odor, cor, odor + cor), realizamos a comparação planejada.

1276 As abelhas *Ptiloglossa* e *X. grisescens* aproximavam e pousavam em flores
1277 artificiais com a cor isolada e cor + odor combinados. Assim, construimos quatro GLMM
1278 com distribuição de Poisson, em cada um dos GLMM avaliamos se havia diferença entre
1279 o número de pousos e aproximações das abelhas *Ptiloglossa* ou *X. grisescens* em cada um
1280 dos tratamentos (cor, cor + odor). Usamos o número de respostas como variável
1281 dependente, as respostas comportamentais (pouso e aproximação) como variável
1282 preditora e a flor como variável aleatória.

1283 Todas as análises foram realizadas com o software Rstudio para Windows. Para
1284 desenvolver os GLMM usamos os pacotes lme4 (Bates et al., 2015). Para fazer a
1285 comparação planejada usamos o multcomp (Hothorn et al., 2008).

1286 **3. RESULTADOS**

1287 **3.1 Composição dos odores florais de *Pseudobombax longiflorum***

1288 As flores exalavam um forte perfume ao longo da antese, percebido pelo nariz
1289 humano. Conforme determinado por SPME e GC-MS, foram encontrados no total 35
1290 compostos de 5 classes químicas nas flores. Os compostos majoritários foram 2-
1291 metilbutanoato de etila, butanoato de etila, 3-metilbutanoato de etila e dissulfeto de
1292 dimetila (Tabela 1).

1293 **Tabela 1.** Quantidades relativas (contribuição de cada composto para o aroma total) dos compostos voláteis orgânicos do perfume floral de
 1294 *Pseudobombax longiflorum*.

Compostos	IR (calculado)	IR (literatura)	ng/flor/h	Contribuição realtiva (%)
Compostos contento enxofre				
dissulfeto de dimetila	769	756	7,38 ($\pm 5,54$)	7,0
Alifático				
2-metilbutanoato de etila	863	854	9,50 ($\pm 13,19$)	24,6
butanoato de etila	818	799	8,80 ($\pm 11,48$)	22,0
3-metilbutanoato de etila	866	859	6,04 ($\pm 10,43$)	19,4
butanoato de propila	903	900	1,74 ($\pm 2,08$)	3,7
(<i>E</i>)-2-metil-2-butenoato de etila	943	938	0,80 ($\pm 0,80$)	1,6
2-metilbutanoato de metila	785	780	0,79 ($\pm 0,80$)	1,4
2-metil-butirato de propila	947	942	0,68 ($\pm 0,74$)	1,4
3-metilbutanoato de propila	950	946	0,44 ($\pm 0,54$)	1
hexanoato de etila	1.000	1.003	0,46 ($\pm 0,54$)	1,0
butirato de isobutila	956	953	0,52 ($\pm 0,44$)	0,8
2-metilbutil butanoato	1.060	1.056	0,37 ($\pm 0,38$)	0,4
<i>n</i> -undecano	1.100	1.100	0,61 ($\pm 0,70$)	0,3
solusterol	1.109	1.109	1,72 ($\pm 2,93$)	0,3
<i>n</i> -hexadecano	1.600	1.600	0,45 ($\pm 0,33$)	0,05
Monoterpeno				
limoneno	1.030	1.030	3,52 ($\pm 4,50$)	3,2
α -pineno	937	933	0,64 ($\pm 0,96$)	1,8
<i>p</i> -cymeno	1.026	1.025	0,57 ($\pm 0,60$)	1,2
3-careno	1.011	1.009	0,78 ($\pm 0,46$)	0,6
β -pineno	978	978	0,37 ($\pm 0,27$)	0,4
(<i>E</i>)- β -ocimeno	1.049	1.050	0,38 ($\pm 0,50$)	0,3
Sesquiterpeno				
α -copaeno	1.386	1.375	1,27 ($\pm 0,83$)	0,9
β -bourboneno	1.396	1.385	0,34 ($\pm 0,27$)	0,5

α -humuleno	1.469	1.469	0,33 ($\pm 0,34$)	0,4
(<i>E</i>)- β -caryofileno	1.434	1.424	0,22 ($\pm 0,17$)	0,4
α -cubebeno	1.359	1.349	0,16 ($\pm 0,08$)	0,2
γ -muuroleno	1.491	1.490	0,21 ($\pm 0,25$)	0,1
zonareno	1.538	1.537	0,27 ($\pm 0,34$)	0,1
β -copaeno	1.443	1.437	0,04 ($\pm 0,03$)	0,06
γ -cadineno	1.530	1.529	0,14 ($\pm 0,11$)	0,05
germacreno D	1.459	1.459	0,42 ($\pm 0,91$)	0,04
Aromático				
benzoato de etila	1.180	1.175	1,92 ($\pm 1,53$)	1,4
benzaldeído	960	960	0,99 ($\pm 1,59$)	0,4
Homoterpeno				
(<i>E</i>)-4,8-dimetil-nona-1,3,7-triene	1.119	1.113	0,20 ($\pm 0,19$)	0,2

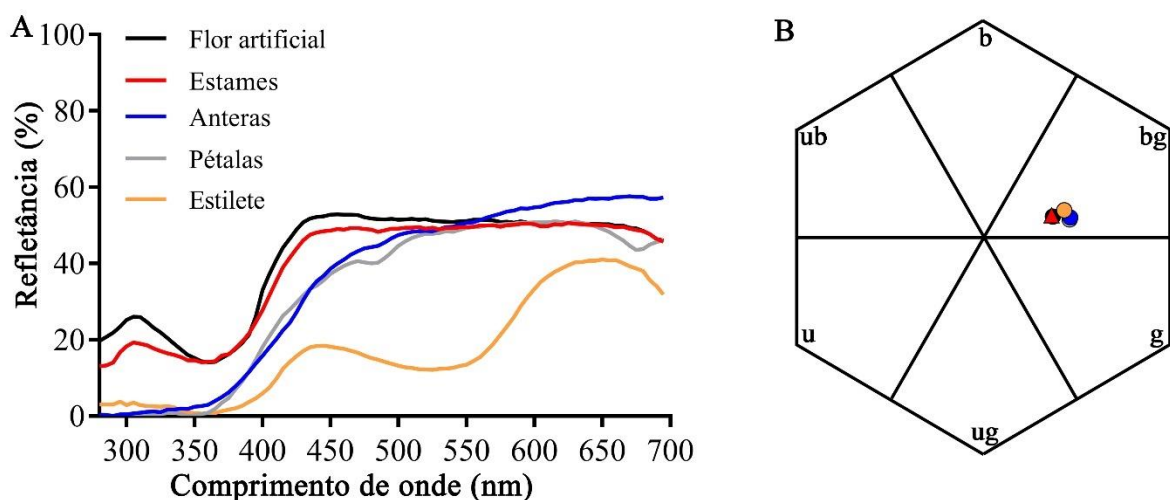
1295 **Legenda:** IR: índice de retenção. Os compostos majoritários (> 5%) são indicados em negrito.

1296 .

1297 3.2 Refletância espectral e hexágono de cor das flores naturais e artificiais

1298 Medições de refletância dos filetes brancos (Figura 1A) revelaram um amplo espectro
 1299 com pico na região do UV (300 – 400 nm) e platô na região que se estende do azul ao vermelho
 1300 (400 – 700 nm) (Figura 2A). Já a porção abaxial das pétalas e as anteras apenas refletem luz do
 1301 azul ao vermelho, possuindo coloração branca levemente amarelada aos olhos humanos (Figura
 1302 1A), graças à maior refletância na região do verde-vermelho (Figura 2A). O espectro de
 1303 refletância do estilete, cuja coloração aos olhos humanos é magenta (Figura 1A), apresenta
 1304 picos nas regiões do azul (~ 440nm) e do vermelho (~ 650nm, Figura 2A). Considerando que
 1305 os filetes constituem a maioria da superfície floral de *P. longiflorum* (Figuras 1A-B),
 1306 selecionamos para confecção das flores artificiais um papel branco com padrão equivalente de
 1307 refletância espectral entre 300 e 700 nm (Figura 2A).

1308 De acordo com o modelo hexagonal para a visão tricromática de abelhas, a cor branca dos
 1309 filetes e das flores artificiais é praticamente equivalente para as abelhas ($\Delta S = 0,009$; Figuras
 1310 2B). Além disso, a cor das outras estruturas florais como anteras, pétalas e estilete também são
 1311 equivalentes para as abelhas, praticamente se sobrepondo no modelo hexagonal ($\Delta S \leq 0,05$;
 1312 Figuras 2B). Contudo, segundo o modelo de hexágono a cromaticidade dos estames difere das
 1313 pétalas e anteras para os olhos das abelhas ($\Delta S \geq 0.9$).



1314

1315 **Figura 2.** Refletância espectral e distâncias perceptuais de estruturas florais de *Pseudobombax*
 1316 *longiflorum* e da flor artificial. A) Espectros de refletância relativa média da flor artificial,
 1317 filetes, anteras (com pólen), pétalas e estilete. B) Loci de cor das estruturas florais de
 1318 *Pseudobombax longiflorum* e da flor artificial no modelo de hexágono para himenópteros
 1319 tricromáticos. Os cantos dos hexágonos representam a estimulação dos três tipos de

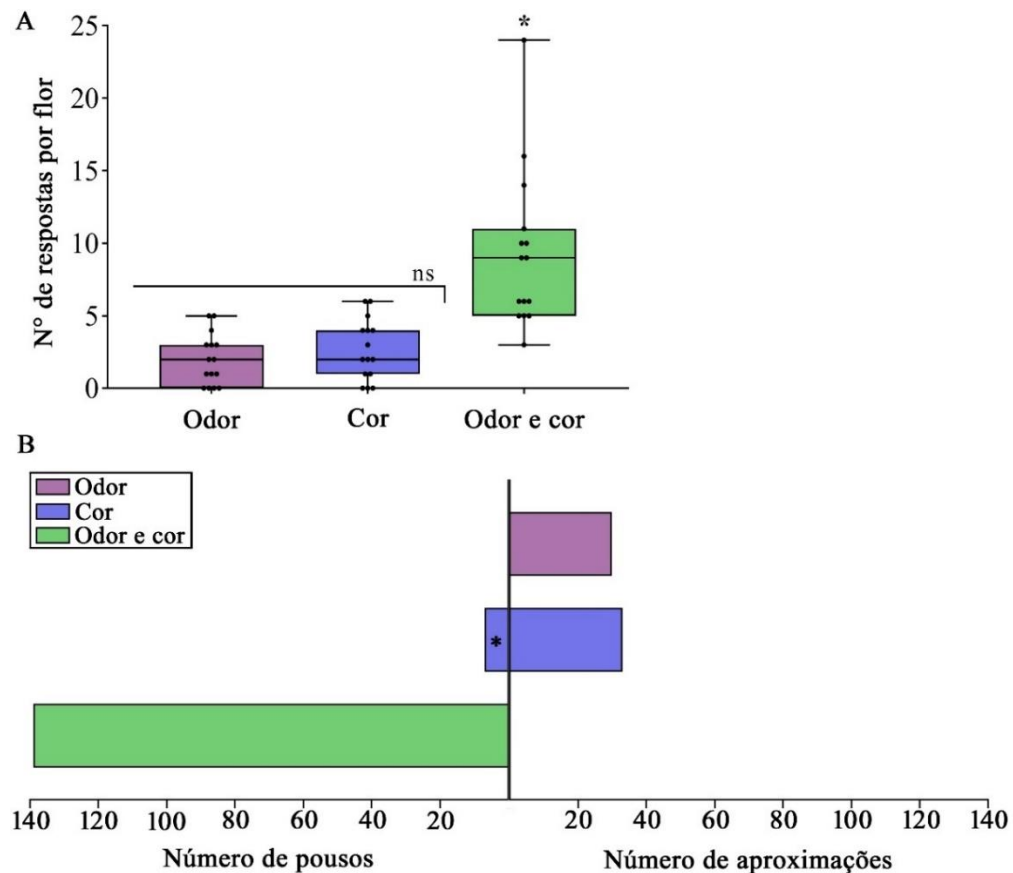
1320 fotorreceptores de abelhas para verde (g), ultravioleta (u) e azul (b), e possíveis combinações
1321 entre eles (ub, ug, bg). O centro da figura representa a refletância do fundo acromático para as
1322 estruturas florais (flor artificial = círculo preto, filetes = triângulo vermelho, anteras = círculo
1323 azul, pétalas = círculo cinza, estilete = círculo laranja). A distância entre os pontos indica o
1324 quão semelhantes as cores são para as abelhas

1325 **3.3 Bioensaios**

1326 **3.3.1 Flores naturais**

1327 O número de respostas totais (aproximações + pouso) das abelhas crepusculares diferiu
1328 entre as flores de *P. longiflorum* com o odor e cor isolados e combinados ($\chi^2 = 97,0$; gl= 2; p<
1329 0,001; figura 3A). As abelhas *Ptiloglossa* responderam com mais frequência às flores com odor
1330 e cor combinados do que as flores com o odor (p< 0,001) ou a cor (p< 0,001) isolada. O número
1331 de respostas totais das abelhas crepusculares as flores com o odor ou a cor isolada foram
1332 similares (p= 0,44; figura 3A). As abelhas *Ptiloglossa* não visitaram flores com odor e cor
1333 ausentes.

1334 As abelhas crepusculares apenas aproximavam das flores de *P. longiflorum* com o odor
1335 isolado, mas nunca pousavam (figura 3B). Flores com a cor isolada, por outro lado,
1336 desencadearam mais aproximações (82,5%) do que pousos (17,5%) nessas abelhas ($\chi^2 = 18,3$;
1337 gl= 1; p< 0.001; figura 3B). Já as flores com o odor e cor combinados desencadearam apenas
1338 pousos nas abelhas *Ptiloglossa* (figura 3B).



1339

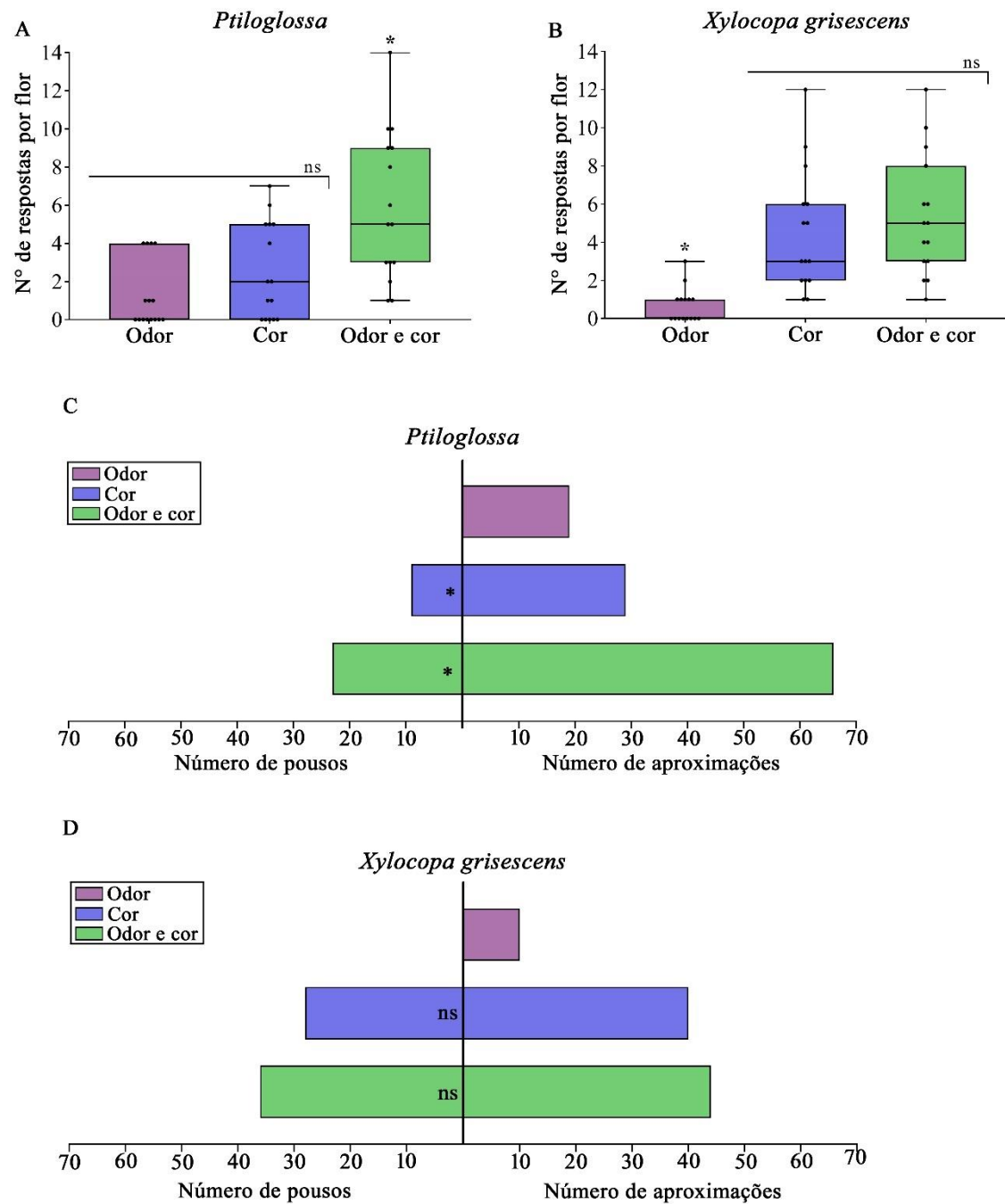
1340 **Figura 3.** Resposta das abelhas *Ptiloglossa* as flores de *P. longiflorum* manipuladas. A) O
 1341 boxplot indica a mediana (linha contínua) e a dispersão (quartis inferior, superior e outliers) do
 1342 número de respostas totais (pousos + aproximações) das abelhas crepusculares às flores com
 1343 odor e cor isolados e combinados. B) Número de pousos (à esquerda) e aproximações (à direita)
 1344 das abelhas *Ptiloglossa* às flores com odor e cor isolados e combinados. Barra violeta = odor
 1345 isolado, barra azul = cor isolada, barra verde = odor e cor combinados. ns = não há diferença
 1346 estatística. Asterisco (*) indica $p < 0,05$.

1347 3.3.2 Flores artificiais

1348 O número de respostas totais (aproximação + pouso) das abelhas *Ptiloglossa* e *X.*
 1349 *grisescens* as flores artificiais foram similares ($\chi^2 = 0,47$; $gl = 1$; $p = 0,49$). Tanto para *Ptiloglossa*
 1350 como para a abelha carpinteira houve diferença no número de respostas totais às flores artificiais
 1351 com odor e cor isolados e combinados ($\chi^2 = 33,8$; $gl = 2$; $p < 0,0001$), porém a frequência que
 1352 cada espécie respondeu a estas flores foi diferente ($\chi^2_{\text{espécie} * \text{flores}} = 11,4$; $gl = 2$; $p = 0,003$). As
 1353 abelhas crepusculares mostraram preferência por flores com odor e cor combinados do que
 1354 flores com odor ($p < 0,001$) ou cor ($p < 0,001$) isoladas (figura 4A). O número respostas totais

1355 das abelhas *Ptiloglossa* às flores artificiais com o odor e a cor isoladas não diferiu ($p= 0,99$;
1356 figura 4A). Por outro lado, o número de respostas totais das *X. grisescens* entre flores com odor
1357 e cor combinados e flores com a cor isolada não diferiu ($p= 0,33$; figura 4B). Nestas flores, o
1358 número de respostas totais das abelhas carpinteiras foram maiores do que nas flores com o odor
1359 isolado ($p< 0,001$; figura 4B). As abelhas *Ptiloglossa* e *X. grisescens* não aproximaram ou
1360 pousaram das flores com odor e cor ausentes.

1361 Flores artificiais com o odor isolado desencadearam exclusivamente aproximações tanto
1362 nas abelhas *Ptiloglossa* como em *X. grisescens* (figura 4C-D). Por outro lado, flores artificiais
1363 com a cor isolada desencadearam nas abelhas crepusculares mais aproximações do que pousos
1364 ($\chi^2= 18,3$; $gl = 1$; $p< 0,001$; figura 4C). Contudo, estas mesmas flores desencadearam o mesmo
1365 número de aproximações e pousos em *X. grisescens* ($\chi^2= 2,1$; $gl= 1$; $p= 0,14$; figura 4D). Nas
1366 flores artificiais com odor e cor combinados, as abelhas *Ptiloglossa* aproximaram mais do que
1367 pousaram ($\chi^2= 21,6$; $gl = 1$; $p< 0,001$; figura 4C). Enquanto, nestas mesmas flores, não houve
1368 diferença estatística no número de aproximações e pousos pelas abelhas carpinteiras ($\chi^2= 0,8$;
1369 $gl= 1$; $p= 0,37$; figura 4D).



1370

1371 **Figura 4.** Respostas das abelhas *Ptiloglossa* e *X. grisescens* ao bioensaio com flores artificiais.
 1372 A - B) Os boxplots indicam a mediana (linha sólida) e dispersão (quartis inferior, superior e
 1373 outliers) das respostas totais (pouso + aproximação) das abelhas *Ptiloglossa* (A) e *X. grisescens*
 1374 (B) as flores artificiais com odor e cor isolados e combinados. C - D) Número de pousos (à
 1375 esquerda) e aproximação (à direita) das abelhas *Ptiloglossa* (C) e *X. grisescens* (D) em flores
 1376 artificiais com odor e cor isolados e combinados. Barra violeta = odor isolado, barra azul = cor

1377 isolada, barra verde = odor e cor combinados. ns = indica que não há diferença estatística.
1378 Asterisco (*) indica $p < 0,05$.

1379 4. DISCUSSÃO

1380 Nosso estudo revelou que as abelhas usam tanto os odores quanto a cor das flores de *P.*
1381 *longiflorum* como pistas para localizá-las durante o amanhecer. Os odores florais guiam as
1382 abelhas até as flores, mas esta pista isolada não induz a visita floral. A cor, contudo, parece ser
1383 importante para desencadear o pouso e a coleta de recurso pelas abelhas. Embora as abelhas
1384 crepusculares *Ptiloglossa* tenham sido capazes de localizar flores com o odor ou a cor isolada,
1385 o número de respostas aumentou quando ambas as pistas estavam combinadas. Esse resultado
1386 mostra que os odores e cores são igualmente importantes na busca de flores por essas abelhas.
1387 A espécie diurna *Xylocopa grisescens*, contudo, usou com mais frequência a cor do que os
1388 odores como pista para encontrar as flores de *P. longiflorum*. Além disso, para *X. grisescens* a
1389 combinação das pistas não torna a flor mais atrativa do que flores com a cor isolada. Esse
1390 resultado indica que durante o forrageamento, as abelhas carpinteiras usam preferencialmente
1391 a cor das flores de *P. longiflorum* como pista para encontrá-las.

1392 Vários estudos destacam a importância dos odores florais como pistas para atrair abelhas
1393 crepusculares para encontrar flores no crepúsculo (Carvalho et al., 2012; Knoll e Santos, 2012;
1394 Cordeiro et al., 2017, Krug et al., 2018, Martinez-Martinez et al., 2021). Aqui, demonstramos
1395 que os odores guiam as abelhas até as flores, mas que esta pista sozinha não desencadeia a visita
1396 floral. Para o pouso, parece ser necessário o estímulo visual. Abelhas crepusculares dos gêneros
1397 *Megalopta* e *Ptiloglossa* são atraídas a compostos florais sintéticos liberados por papel filtro
1398 (Cordeiro et al., 2017, Krug et al., 2018, Martinez-Martinez et al., 2021). Nestes estudos, os
1399 autores não discriminaram entre o número de pousos ou aproximações das abelhas. Além disso,
1400 não foi possível saber se as abelhas usavam a coloração clara do papel filtro como pista de
1401 orientação. Abelhas *Megalopta* também são atraídas pelos compostos usados em armadilhas
1402 para machos de Euglossini, mas neste método não há observação do comportamento das abelhas
1403 (Carvalho et al., 2012; Knoll e Santos, 2012). Dessa forma, esse é o primeiro estudo a demonstrar
1404 claramente o papel dos odores florais na busca das abelhas crepusculares por flores.

1405 Os compostos majoritários que compõem o buquê floral de *P. longiflorum*, 2-
1406 metilbutanoato de etila, butanoato de etila e 3-metilbutanoato de etila, também são produzidos
1407 por flores polinizadas por besouros, como nas espécies do gênero *Magnolia* (Azuma et al.,

1408 1997), nas espécies *Anaxagorea brevipes* e *Anaxagorea dolichocarpa* (Annonaceae) (Jürgens
 1409 et al., 2000), e por flores polinizadas por moscas como *Sauromatum* (Araceae) (Hadacek e
 1410 Weber, 2002). Já o dissulfeto de dimetila é um composto comum em flores polinizadas por
 1411 morcegos (Dobson, 2006). Além de atrair morcegos, ele também atrai moscas e besouros às
 1412 flores (Borg-Karlson et al., 1993; Bestmann et al., 1997; von Helversen et al., 2000; Hadacek e
 1413 Weber, 2002). Seria interessante saber se as abelhas *Ptiloglossa* responde fisiologicamente e
 1414 comportamentalmente a cada um desses voláteis específicos previamente associados à atração
 1415 de outros visitantes florais.

1416 Em geral, abelhas diurnas utilizam estímulos visuais a longa distância para navegação e
 1417 a curta distância para reconhecimento floral (Lehrer 1996; de Ibarra et al., 2015). Pontos de
 1418 referência na paisagem, posição do sol e padrão de luz polarizada são exemplos de estímulos
 1419 visuais usados no contexto de navegação, enquanto cores e padrões são pistas importantes para
 1420 o reconhecimento de uma flor recompensadora (Lehrer 1996; Rossel e Wehner, 1984;
 1421 Srinivasan, 2010; van der Kooi et al., 2019). Pistas visuais formadas pelo contraste do dossel
 1422 com o céu e ao redor do ninho são pontos de referências importantes durante a navegação e
 1423 orientação de abelhas crepusculares da espécie *Megalopta genalis* (Chaib et al., 2021, Warrant
 1424 et al., 2004). No presente estudo, demonstramos pela primeira vez, em uma espécie de abelha
 1425 crepuscular, o uso de estímulos visuais no comportamento de orientação até a flor. Algumas
 1426 características visuais das flores de *P. longiflorum* podem ter facilitado sua detecção pelas
 1427 abelhas, mesmo em baixa luminosidade. Essas são flores grandes com uma forma de pincel,
 1428 cuja área aumenta à medida que os filetes se afastam durante a antese (Araújo et al. 2021).
 1429 Estudos demonstram que quanto maior é o tamanho de uma flor, mais rápido e eficiente é o
 1430 forrageamento das abelhas na mesma (Spaethe et al., 2001).

1431 Além disso, nosso estudo revelou que os proeminentes filetes brancos de *P. longiflorum*
 1432 possuem um amplo espectro de refletância do ultravioleta ao vermelho, estimulando assim
 1433 todos os três tipos de fotorreceptores das abelhas (Chittka et al., 1994). Este padrão de
 1434 refletância ($uv+ b+ g+ r+$) é raro na natureza (Chittka et al., 1994) e parece maximizar a
 1435 refletância de fótons na paisagem, facilitando a detecção floral em baixa luminosidade (Kelber
 1436 et al., 2003). Curiosamente, esse padrão raro de refletância foi descrito principalmente em flores
 1437 visitadas por esfingídeos noturnos (Chittka et al. 1994) porém um estudo recente identificou
 1438 padrão similar em flores de *Machaerium opacum*, uma espécie melitófila polinizada por abelhas
 1439 crepusculares (Siqueira et al., 2018). Segundo modelo hexagonal de percepção cromática de

1440 abelhas, as anteras e as pétalas diferem-se cromaticamente e se contrastam dos filetes das flores
1441 de *P. longiflorum*. Este contraste entre as estruturas florais também é provavelmente usado por
1442 abelhas no reconhecimento, encontro de recursos e/ou manipulação da flor.

1443 De fato, ambas as espécies de abelha estudadas pousaram em flores apresentando apenas
1444 estímulos visuais, indicando que essas pistas possuem um papel importante na coleta do recurso
1445 floral. Apesar disso, nosso estudo mostra que pistas visuais possuem maior importância no
1446 reconhecimento floral para a abelha diurna *X. grisescens* do que para a abelha crepuscular
1447 *Ptiloglossa*. Flores artificiais que simulavam apenas o padrão visual dos filetes desencadeou
1448 poucos pousos em *Ptiloglossa*, enquanto o número de pousos e aproximações das abelhas
1449 carpinteiras nessas mesmas flores foi equivalente. Inclusive, em vários pousos observamos as
1450 abelhas carpinteiras raspando as extremidades apicais das flores artificiais com as pernas,
1451 comportamento similar ao descrito para essas abelhas ao coletarem pólen das flores naturais de
1452 *P. longiflorum* (Araújo et al., 2021). Estudos complementares são ainda necessários para melhor
1453 entender o papel da cor, da forma, do tamanho e de contrastes na atração de abelhas
1454 crepusculares e diurnas por flores de *P. longiflorum*. É possível que esses estímulos visuais
1455 atuem de forma distinta na detecção das flores as longas distâncias e/ou reconhecimento e
1456 manipulação de estruturas florais a curtas distâncias.

1457 A importância das pistas florais varia entre os diferentes sistemas de polinização, porém
1458 a maioria dos estudos observou que mesmo sendo capazes de encontrar suas flores hospedeiras
1459 apenas com as pistas visuais ou olfativas, abelhas diurnas sociais ou solitárias oligoléticas são
1460 mais atraídas quando estas pistas são combinadas (Kulahci et al., 2008; Burguer et al., 2010;
1461 Dötterl et al., 2011; Milet-Pinheiro et al., 2012; Dötterl et al., 2014; Milet-Pinheiro et al., 2016;
1462 Lawson et al., 2018; Rachersberger et al., 2019). Embora este mesmo efeito sinérgico tenha
1463 sido observado no presente estudo para abelhas crepusculares *Ptiloglossa*, a combinação de
1464 pistas visuais e olfativas não aumentou a atratividade da flor para abelhas carpinteiras em
1465 relação a flores apresentando apenas estímulos visuais. Essa diferença observada no
1466 comportamento de *X. grisescens* em relação a outras abelhas diurnas pode estar relacionada aos
1467 voláteis que compõem o buquê floral de *P. longiflorum*, que não são comuns nas flores
1468 melitófilas, e assim, talvez causem menor atração. Além disso, as abelhas carpinteiras visitam
1469 as flores apenas quando há um aumento nos níveis de luz no ambiente (Araújo et al., 2021).
1470 Dessa forma, a quantidade de luz no ambiente pode ser o suficiente para produzir um sinal
1471 visual seguro (Warrant, 2017), tornando a cor e/ou forma dos filetes uma pista confiável para

1472 detecção das flores. Por outro lado, abelhas crepusculares *Ptiloglossa* visitam as flores quando
 1473 os níveis de luz no ambiente são duas ordens de magnitude menores do que no caso de *X.*
 1474 *grisescens* (Araújo et al., 2021). Neste contexto, demonstramos aqui que a integração de
 1475 estímulos visuais e olfativos pelas abelhas crepusculares facilita o reconhecimento e a
 1476 exploração de flores de *P. longiflorum* em baixa luminosidade.

1477

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CAPÍTULO III**BODY SIZE AND THE ARCHITECTURE OF VISUAL ORGANS IN
CREPUSCULAR AND DIURNAL PHYLOGENETICALLY RELATED BEES**

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1695 **ABSTRACT**

1696 Diurnal bees have the size of visual structure related to body size. On the other hand,
1697 crepuscular bees have large compound eyes, facets, and ocelli in relation to their body size.
1698 Studies indicate that the larger size of these visual structures is an adaptation to forage in low
1699 light environments. However, it is not clear whether the sizes of visual structures of crepuscular
1700 bees are related only by temporal patterns or also by their body size. Furthermore, the visual
1701 adaptations of crepuscular bees were described mainly in *Megalopta genalis*, while little is so
1702 far known about other crepuscular bees and the influence of phylogenetic relationships in this
1703 system. Here we evaluated how body size relates to the architecture of the visual structures of
1704 crepuscular and diurnal bee representatives of the families Halictidae and Colletidae. After
1705 analyzing six distinct morphometric variables in the visual organs of 11 bee species, we also
1706 tested whether variations in these attributes were related to the temporal patterns (crepuscular
1707 or diurnal) and/or the phylogenetic relationships. The six visual attributes measured in diurnal
1708 and crepuscular bees of distinct body sizes (intertegular distance varying from 1.7 to 5.2 mm)
1709 were the size of the central ocellus, compound eyes, dorsal, frontal, and ventral ommatidia, and
1710 the eye ommatidial density. We found that these variables were generally related by both body
1711 size and the temporal pattern of the bee. However, the eye ommatidial density and the diameter
1712 of the frontal ommatidia of crepuscular bees were not correlated with their body sizes. These
1713 two variables presented equivalent values in crepuscular bees of distinct body sizes (2.0 to 5.2
1714 mm). The lower variability in the mean ommatidial density of crepuscular bees is probably
1715 related to the existence of an ideal threshold between light sensibility and visual acuity, as an
1716 adaptation to dim light environments. Our study revealed that the frontal region of the
1717 compound eyes, which is particularly important for ecological interactions such as flower
1718 recognition, present conserved sizes in crepuscular bees of distinct body sizes. In other words,
1719 although small crepuscular individuals were selected during the evolutionary transition of
1720 temporal niche, these individuals conserved larger frontal ommatidia. Furthermore, bees
1721 sharing the same temporal pattern (crepuscular or diurnal) presented more similarity in their
1722 visual organ traits than bees of different temporal patterns sharing a same taxon.

1723 **KEY-WORDS:** crepuscular bees, nocturnal bee, facultative crepuscular bees, diurnal bees,
1724 visual system, apposition compound eyes.

1725

1726 1. INTRODUCTION

1727 During the day, terrestrial and celestial cues are easily seen and used by diurnal insects
1728 for orientation in contexts like foraging, searching for mating partners or navigating back to the
1729 nest (Von Frisch 1974; Wehner 1984; el Jundi et al., 2014). Throughout the night, the moon
1730 and stars are the only source of natural light, and nocturnal insects use this low light levels for
1731 doing the same tasks that diurnals do (Dacke et al., 2003; Hironaka et al., 2007; Narendra et al.,
1732 2013; Warrant et al., 2004). Two adaptive hypotheses have been raised to explain the
1733 advantages of foraging at crepuscular or nocturnal environments. First, these environments
1734 offer a niche with fewer natural enemies than the diurnal (Wcislo et al., 2004). Second, they
1735 present, in general, fewer competitors (Kerfoot 1967; Kelber et al., 2006; Wcislo et al., 2004;
1736 Smith et al., 2017, Araújo et al., 2021). However, there are also disadvantages, as the few
1737 photons available in dim-light environments can make vision less reliable, bringing challenges
1738 to navigation and foraging (Warrant 2017). In this framework, insects owning compound eyes
1739 with higher sensitivity to light, as well as neural adaptations within the retina and optic lobes
1740 were selected to occupy the crepuscular and nocturnal environments (Greiner et al., 2004a;
1741 Warrant et al., 2004; Yilmaz et al., 2014; O'Carroll and Warrant 2017; Warrant 2017). These
1742 specializations increase the visual signal-to-noise ratio, making the vision more reliable in low
1743 light (Warrant 2017).

1744 The compound eyes of insects are formed by many optic units called ommatidium
1745 (Nilsson 1989). Each ommatidium possesses a corneal lens (facets) and a crystalline cone
1746 responsible for focusing the incident light on the rhabdom (Nilsson 1989). The rhabdom is the
1747 light-sensitive portion of the photoreceptor cells and is composed of microvilli where the
1748 photosensitive molecules are located (Nilsson 1989). Each photoreceptor in the insect retina is
1749 responsible for detecting only a small region of the visual field, corresponding to little details
1750 of the scene (Warrant 2017). Therefore, compound eyes with increased spatial acuity tend to
1751 have higher amounts of photoreceptor per retinal area, as a consequence of smaller and more
1752 densely distributed ommatidia (Snyder 1979). Yet, the amount of light that reaches an
1753 ommatidium declines proportionally to the diameter of its lens (Snyder 1979). Thus, there is a
1754 trade-off between resolution and light sensitivity (Snyder 1979). The eyes of diurnal insects,
1755 that are active in high light intensities (e.g., 10^4 cd m⁻²), have smaller lens diameter and higher
1756 density of ommatidia per area (O'Carroll and Warrant 2017). Consequently, more details of the

1757 scene are detected, which improves spatial resolution (Yilmaz et al., 2014; Cronin et al., 2014).
1758 Conversely, insects that are active during dim-light, when illumination levels can be smaller
1759 than 1 cd m^{-2} , have larger lens diameter and lower ommatidial density in their compound eyes
1760 (Yilmaz et al., 2014; O'Carroll and Warrant 2017). These adaptations make the eyes of
1761 crepuscular and nocturnal insects more sensitive to light but reduce their spatial resolution
1762 (Cronin et al., 2014).

1763 Two main types of compound eyes are characterized in insects: apposition and
1764 superposition eyes (Land 1992). The main difference between these types of eyes is the number
1765 of lens units that focus light on a single rhabdom (Cronin et al., 2014). In apposition eyes, the
1766 light that reaches each rhabdom comes from just one lens, while in superposition eyes, the light
1767 comes from many lenses (Nilsson 1989). The last type of eye is suited to high optical sensitivity
1768 (Warrant and Dacke 2011). Although superposition compound eyes are more adapted to dim-
1769 light vision, crepuscular bees have apposition compound eyes (Menzi 1987; Warrant et al.,
1770 2004; Greiner et al., 2004b).

1771 The habit of foraging during the twilight has presumably evolved 19 times among bees
1772 (Wcislo and Tierney 2009), and occurs in four of the seven bee families: Halictidae, Colletidae,
1773 Andrenidae, and Apidae (Michener 2007). This temporal pattern of foraging activity is defined
1774 as crepuscular and can be distinguished from two other temporal patterns in bees: crepuscular
1775 facultative and nocturnal (Wcislo and Tierney, 2009). *Xylocopa tranquebarica*, for instance, is
1776 considered nocturnal, since it can forage in moonless nights (Somanathan et al., 2009). In this
1777 condition, light available is around 100 million times lower and much redder than on a sunny
1778 day (O'Carroll and Warrant 2017). In contrast, *Ptiloglossa*, *Megommation*, *Zikanapis*,
1779 *Megalopta*, *Xenoglossa*, and some *Xylocopa* are considered as crepuscular bees (Linsley 1958;
1780 Hurd and Linsley 1964; Michener 1966; Cordeiro et al., 2017; Janzen 1968; Kelber et al., 2006;
1781 Somanathan et al., 2009), since they forage during the nightfall or when the first rays of light
1782 reach the earth during the sunrise (Warrant et al., 2004, Kelber et al., 2006; Liporoni et al.,
1783 2020). In these periods, light available is around 100 times less intense than in a cloudless day
1784 (O'Carroll and Warrant 2017). Other bees within the genus *Caupolicana* are crepuscular
1785 facultative, meaning that these species occasionally forage during the twilight but usually are
1786 active during the day (Linsley and Cazier, 1970; Wcislo and Tierney 2009).

1787 Visual adaptations for navigating and foraging at low light were mainly described in
1788 *Megalopta genalis* (Halictidae: Augochlorini), the most studied crepuscular bee at the
1789 physiological level. This species has large eyes relative to its body size (Jander and Jander
1790 2002), large facets diameter, and wide and long rhabdoms when compared to diurnal bees *Apis*
1791 *mellifera* (Apidae: Apini) and *Lasioglossum leucozonium* (Halictidae: Halictini) (Greiner et al.,
1792 2004a; Warrant et al., 2004). These adaptations increase light capture (Warrant et al., 2004).
1793 Another adaptation usually related to nocturnal lifestyle is the large ocelli relative to its body
1794 size (Kerfoot 1967; Warrant et al., 2006; Wcislo and Tierney 2009). The function of the three
1795 dorsal ocelli present in the bee head is not yet well established, but a larger diameter of these
1796 structures is a good indicator of crepuscular habit and could be related to an increased need of
1797 light sensitivity (Kerfoot 1967; Warrant et al., 2006; Warrant 2007).

1798 Although the bodies of crepuscular bees of distinct species vary in size, most of them
1799 present larger body sizes than their related diurnal taxa (Wcislo and Tierney 2009). Larger bees
1800 tend to have proportionally larger compound eyes and facets that enhance overall visual
1801 sensitivity, thus large body size might be a pre-adaptation to the evolution of dim-light foraging
1802 (Wcislo and Tierney 2009). Diurnal bees present proportional and directly-related sizes of
1803 visual structures and body (Jander and Jander et al., 2002; Spaethe and Chittka 2003;
1804 Kapustjanskij et al., 2007; Streinzer et al., 2016). However, the relationship between optical
1805 features and body size to crepuscular bees is so far unclear. Some studies suggest that the larger
1806 size of visual structures in relation to body sizes found in crepuscular bees could be a result of
1807 selection pressure to explore low light environments (Jander and Jander 2002; Warrant et al.,
1808 2004; Somanathan et al., 2009). Contrarily, other works propose that *crepuscular bees* present
1809 a correlation between body size and the size of both eyes and ocelli, whereas in nocturnal bees,
1810 body sizes only appear to correlate with the eye, but not with the ocelli size (Kelber *et al.*,
1811 2006).

1812 Here we aimed understand whether and how the size of visual structures of different
1813 diurnal and crepuscular bee species is related to their body sizes and/or temporal patterns of
1814 foraging. We thus evaluated the size of the bodies, ocelli, compound eyes and ommatidia along
1815 the eye surface, as well as the mean ommatidial density, in phylogenetically related bees of
1816 Halictidae and Colletidae families. We then tested how the variations between these attributes
1817 measured in the visual systems of crepuscular, facultative crepuscular and diurnal bees were

1818 related to their temporal patterns and/or to phylogenetic relationships. We discuss our data in
1819 light of how bees with distinct temporal patterns resolve the trade-off between sensitivity to
1820 light and spatial resolution at the level of their visual organs.

1821

1822 **2. MATERIAL AND METHODS**

1823 We evaluated the external morphology of the visual organs of bees of different temporal
1824 patterns (crepuscular, crepuscular facultative, and diurnal), body sizes (1.7 to 5.2 mm) and
1825 families (Halictidae and Colletidae). All specimens used in this study were females, to avoid
1826 the influence of sexual dimorphism.

1827 **2.1 Bee Species**

1828 Morphometric analyses were performed in 68 specimens across 11 species and two
1829 families (Table 1, Figure 1). Bee specimens were acquired from the Entomological Collections
1830 Paulo Nogueira Neto (CEPPANN) and Prof. J.M.F. Camargo (FFCLRP-USP) of the University
1831 of São Paulo, and Taxonomic Collection Center of the Federal University of Minas Gerais
1832 (CCT-UFMG), Brazil.

1833 To allow relevant comparison between phylogenetically related species within a family
1834 and evaluation of adaptive phenomena in distinct families, we selected representatives of
1835 crepuscular and diurnal bees of Halictidae and Colletidae. We considered the phylogenies
1836 published by Gonçalves (2016) for Halictidae, and Almeida and Danforth (2009) for Colletidae.
1837 In Halictidae, the Augochlorini tribe has three genera identified as crepuscular (Gonçalves,
1838 2016), from which we selected *Megalopta* and *Megommation*. As close-related diurnal species
1839 (Table 1), we used *Augochlora esox* (Augochlorini) and *Pseudoaugochlora graminea*
1840 (Augochlorini). In Colletidae (Table 1), we used the crepuscular bees *Ptiloglossa latecalcarata*
1841 and *Zikanapis megalopta* (both from tribe Caupolicanini), the facultative crepuscular species
1842 *Caupolicana yarrowi* (tribe Caupolicanini), and the diurnal species *Cadeguala occidentalis*
1843 (tribe Diphaglossini). The bees *Caupolicana yarrowi* and *Cadeguala occidentalis* were
1844 collected in United States and Chile, respectively.

1845 **Table 1.** Bees used for morphologic comparisons and the respective temporal patterns of each species (diurnal, crepuscular or facultative
 1846 crepuscular). n is the number of specimens analyzed.

Temporal patterns		
Diurnal (n)	Crepuscular (n)	Facultative crepuscular (n)
Halictidae: Augochlorini	Halictidae: Augochlorini	Halictidae: Augochlorini
<i>Augochlora (Augochlora) esox</i> (Vachal, 1911) (5)	<i>Megalopta aegis</i> (Vachal, 1904) (14) <i>Megalopta amoena</i> (Spinola, 1853) (9) <i>Megalopta guimaraesi</i> Santos & Silveira, 2009 (3)	none
<i>Pseudoaugochlora graminea</i> (Fabricius, 1804) (9)	<i>Megalopta sodalis</i> (Vachal, 1904) (8) <i>Megommation insigne</i> (Smith, 1853) (7)	none
Colletidae: Diphaglossini	Colletidae: Caupolicanini	Colletidae: Caupolicanini
<i>Cadeguala occidentalis</i> (Haliday, 1836) (2)	<i>Ptiloglossa latealcarata</i> Moure, 1945 (5) <i>Zikanapis Megalopta</i> Moure, 1948 (4)	<i>Caupolicana yarrowi</i> Cresson, 1875 (2)



1847

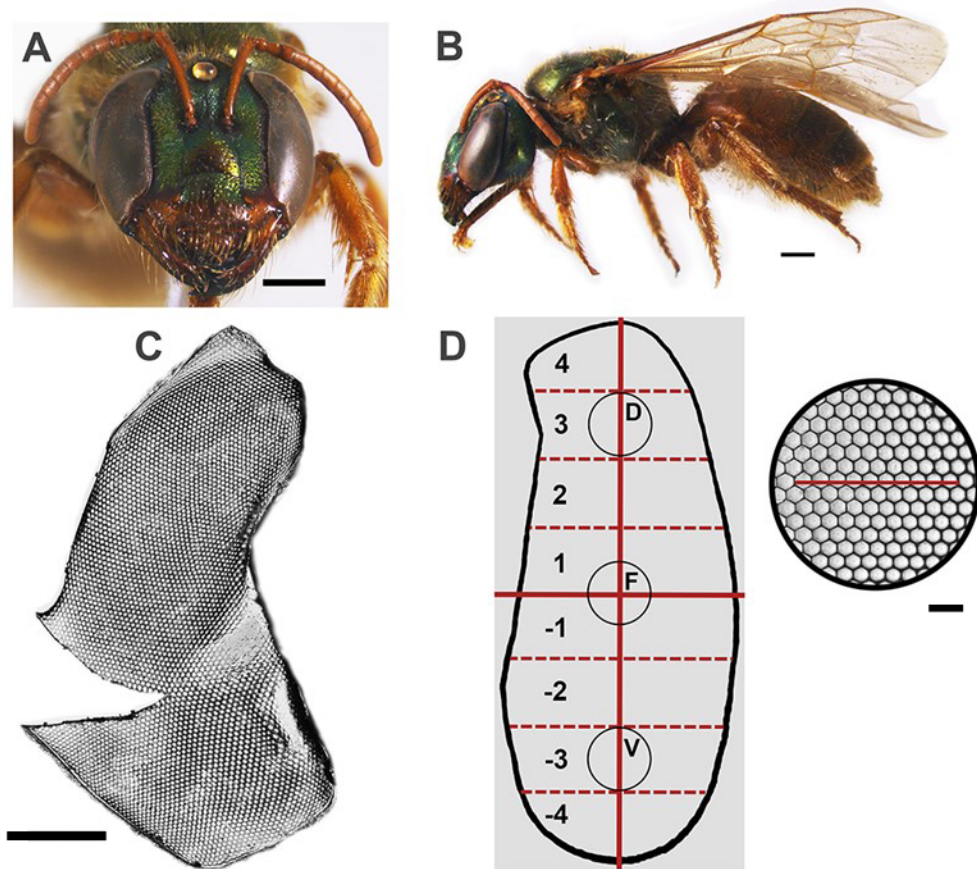
1848 **Figure 1.** Head and visual organs of diurnal and crepuscular bees from Halictidae (A-F) and Colletidae (G-J). A) *Megalopta aegis* (crepuscular);
 1849 B) *Megalopta guimaraesi* (crepuscular); C) *Megalopta amoena* (crepuscular) D) *Megommation insigne* (crepuscular); E) *Augochlora esox*
 1850 (diurnal); F) *Pseudoaugochlora graminea* (crepuscular); G) *Ptiloglossa latecalcarata* (crepuscular); H) *Caupolicana yarrowi* (facultative
 1851 crepuscular); I) *Zikanapis megalopta* (crepuscular); J) *Cadeguata occidentalis* (diurnal) Scale = 1mm.

1852 2.2 Measurements of body size and visual structures

1853 To estimate the body size of each bee, we measured its intertegular distance (Cane 1987)
1854 from a photograph taken with a camera coupled to a stereomicroscope (Luxeo 4D Digital
1855 Stereozoom Microscope, LABOMED), with magnification of 10 times. We photographed the
1856 central ocelli with 100 times magnification for measuring their diameter (Figure 2A). To allow
1857 precise measurements of compound eye structures, we prepared nail polish molds (adapted
1858 from van Praagh et al., 1980) for each specimen studied (Figure 2B-C). After carefully
1859 removing each mold from the compound eye, we made a small incision to flattened it and
1860 prepared a glass slide with a coverslip. Images of the eye molds were taken in a microscope
1861 (DM4000B, Leica) coupled to a camera (DFC425, Leica) with 25 times of amplification.

1862 Images obtained were used to count the total number of ommatidia and measure the eye
1863 area. From these measurements, we calculated the ommatidial density using the equation:
1864 $\text{ommatidia number} / \text{area of a compound eye}$. This equation provides an estimation of compound
1865 eye resolution (Yilmaz et al., 2014). We also measured the diameter of 10 ommatidia in the
1866 dorsal, frontal, and ventral regions of the left compound eye (Figure 2D), the diameter of dorsal
1867 ommatidia was not measured in the dorsal rim area. To measure these regions, we selected ten
1868 ommatidia located after a certain distance the edge of the eye. The distance from the edge of
1869 the eye was of 150 μm until 300 μm according to the size of the bee's eye. Frontal area we
1870 measured ten ommatidia located in the center of the eye. These values were used to calculate
1871 an average diameter for each eye region, considering that ommatidial size was previously
1872 shown to vary across the eye surface of bees (Jander and Jander 2002; Greiner et al., 2004b).

1873 All morphometric data was analyzed using the software Image J (Abramoff et al., 2004).
1874 The function “*contour*” was used to measure the area of the left compound eye. The function
1875 “*straights*” was used to measure the intertegular distance, the diameter of the ocelli and the
1876 diameter of ventral, frontal or dorsal ommatidia in the left compound eye.



1877

1878 **Figure 2.** Morphometric analysis of the visual structures of *Megalopta aegis*. A) Frontal view
 1879 of the head showing the prominent central ocellus. We measured the diameter of this visual
 1880 structure. B) Lateral view of the bee showing its left compound eye. We measured the overall
 1881 area and produced a nail polish mold of this structure. C) Mold of the left eye, from which we
 1882 counted the total number of ommatidia and measured the diameter of ommatidia in distinct eye
 1883 regions. D) Coordinate system used to determine three circular areas in which we measured the
 1884 diameter of ommatidia from the dorsal (D), frontal (F) and ventral (V) eye regions. After tracing
 1885 the dorsoventral (vertical) and the anteroposterior (horizontal) long-axes of the eye (red solid
 1886 lines), we divided the eye in eight sectors (from 4 to -4) along the dorsoventral axis (red dashed
 1887 lines). Areas D, F and V were defined within sectors 3, 1/-1 and -3, according to the scheme.
 1888 We then calculated the average diameter of 10 ommatidia (right inset, red line) in each of these
 1889 areas. Scale bars = 1 mm in a and b; 0.5 mm in c and 70 μ m in d.

1890 2.3 Statistics

1891 To test if the temporal patterns and body size relate in the size on visual structure, we
 1892 used as response variables: diameter of the central ocellus; eye area; ommatidial density and
 1893 diameter of dorsal, frontal and ventral ommatidia. A Linear Mixed Model analysis (LMM) test
 1894 was applied to all response variables. We used species as random variable, and the body size
 1895 (intertegular distance) and temporal patterns (diurnal or crepuscular) as fixed effects (predictor
 1896 variable). For these analyses we excluded the facultative crepuscular bee, *Caupolicana yarrowi*,
 1897 because we only had 2 sample of crepuscular facultative habit. To test whether there was a
 1898 difference between the ommatidial diameter of the three eye regions of the crepuscular
 1899 Halictidae and Colletidae, we performed a linear model. For this, we used the diameter of the
 1900 ommatidia as response variable and eye regions (dorsal, frontal, ventral) as the predictor
 1901 variable. When necessary, we performed the planned comparison among the treatments.

1902 A Principal Component Analysis (PCA) with correlation matrix was performed using
 1903 all visual variables measured in bee species, including the facultative crepuscular bee
 1904 *Caupolicana yarrowi*. This analysis was performed to examine which parameters were more
 1905 relevant to distinguish diurnal, crepuscular and facultative crepuscular bees of different
 1906 families. A cluster analysis was performed to group the species based in the similarity of their
 1907 visual structures. We use the Euclidean distance and the average method to perform this
 1908 analysis.

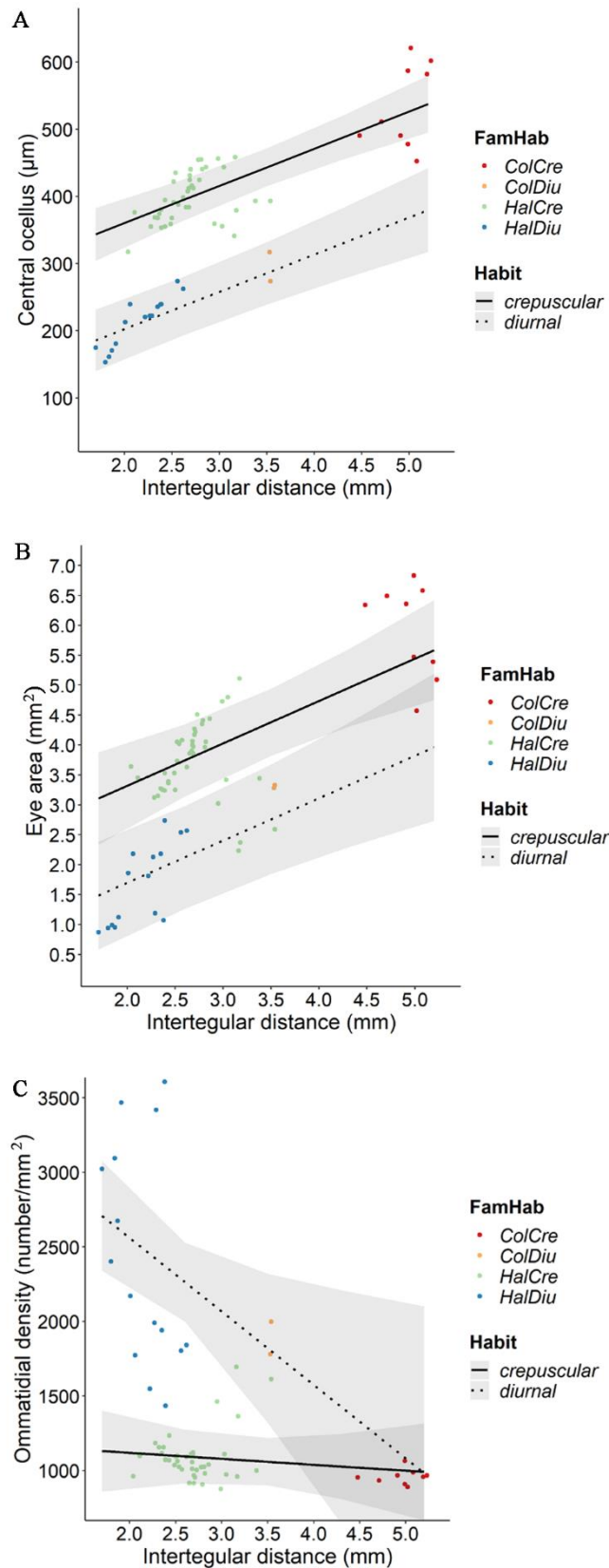
1909 All analyses were performed in software R CRan Project v4 (2020) using the packages:
 1910 tidyverse (Wickham et al., 2019), cluster (Maechler et al., 2019), dendextend (Tal Galili 2015)
 1911 lm4 (Bates et al., 2015), factorextra (Kassambara and Mundt 2020), gridExtra (Auguie 2017),
 1912 ggplot (Wickham 2009), effects (Fox and Weisberg 2019), PerformanceAnalytics (Peterson et
 1913 al., 2018), psych (Revelle 2020), and REdaS (Maier 2015), multcomp (Hothorn et al., 2016).

1914 **3. RESULTS**

1915 In both crepuscular and diurnal bees, the diameter of the central ocellus and the eye area
 1916 were significantly related to the body size (respectively, $F_{(1,27)}=47.6$, $p<0.001$, $F_{(1,27)}=18.3$,
 1917 $p<0.001$; Figure 3A) and the temporal patterns (respectively, $F_{(1,07)}= 35$, $p<0.001$, $F_{(1,7)}=9.47$,
 1918 $p=0.016$; Figure 3B). More precisely, smaller bees had smaller while larger bees had larger
 1919 ocelli and eyes. Also, crepuscular bees had larger ocelli and eyes than diurnal bees (Figure 3A-
 1920 B).

1921 For ocelli and eye area parameters shown in Figures 3 a and b, we did not found
1922 significant interactions between the effects of the body size and the temporal patterns (ocelli:
1923 $F_{\text{body size*temporal patterns}(1.28)}=0.7$, $p=0.40$, eye area: $F_{\text{body size* temporal patterns}(1.27)}=1.3$, $p=0.26$). This
1924 indicates that although diurnal and crepuscular bees significantly differ in those optical
1925 parameters, the way body size relates to them is equivalent in bees of the two temporal patterns.
1926 The equivalent inclination of the solid (crepuscular bees) and the dotted line (diurnal bees) for
1927 each optical parameter reflects such a conclusion (Figure 3A-B).

1928 The average ommatidial density, calculated as the total number of ommatidia divided
1929 by the total eye area, was significantly related to the temporal patterns ($F_{(1.12)}=18.91$, $p<0.001$,
1930 Figure 3C), and to the body size ($F_{(1.17)}=0.8$, $p=0.01$). We also found a significant interaction
1931 between these two effects ($F_{\text{body size* temporal patterns } (1.17)}=4.79$, $p=0.04$). While the ommatidial
1932 density was negatively related to body size in diurnal bees, this parameter did not vary with
1933 body size in crepuscular bees (Figure 3C).



1934

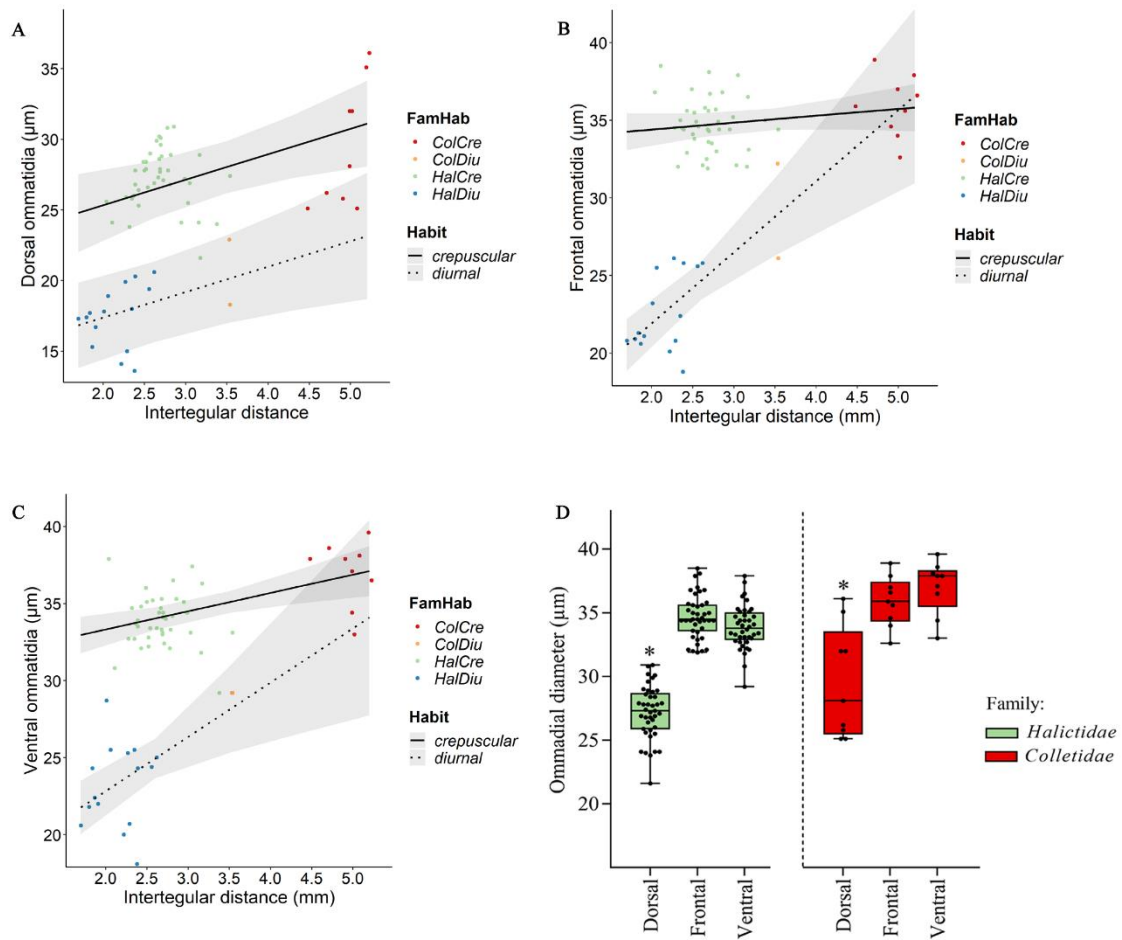
1935 **Figure 3.** Plots from LMM testing the effects of the temporal patterns and the intertegral
 1936 distance (body size) on the following response variables: diameter of the central ocellus (A);

1937 eye area (B); ommatidial density (C). Bold lines indicate crepuscular bees and dotted lines
 1938 indicate diurnal bees. Red dots: Colletidae crepuscular bees; Orange dots: Colletidae diurnal
 1939 bees; Green dots: Halictidae crepuscular bees; Blue dots: Halictidae diurnal bees. The shaded
 1940 areas correspond to the confidence intervals.

1941 Morphometric analysis in distinct eye regions revealed that the diameters of dorsal
 1942 ommatidia (Figure 4A) was significantly related to both body size ($F_{(1,17)}=7.9$, $p=0.01$), and
 1943 temporal patterns of bees ($F_{(1,8)}=20$, $p=0.002$). The diameters of dorsal ommatidia were
 1944 positively associated with the bee size and, additionally, were higher in crepuscular bees than
 1945 in diurnal bees (Figure 4A). No significant interaction was found between the effect of the body
 1946 size and the temporal patterns on the diameter of dorsal ommatidia ($F_{\text{body size} * \text{temporal}}$
 1947 $\text{patterns}_{(1,17)}=0.16$, $p=0.68$).

1948 Unlike dorsal ommatidia, we found not only a significant effect of the body size
 1949 ($F_{(1,23)}=24$, $p<0.001$, $F_{(1,63)}=17$, $p=0.001$, respectively) and the temporal patterns ($F_{(1,15)}=64$,
 1950 $p<0.001$, $F_{(1,63)}=29$, $p<0.001$, respectively) on the diameter of frontal and ventral ommatidia ,
 1951 but also a significant interaction between these two effects ($F_{\text{body size} * \text{temporal patterns}_{(1,23)}}=16$,
 1952 $p<0.001$, $F_{\text{body size} * \text{temporal patterns}_{(1,63)}}=4.2$, $p=0.045$, figures 4B and 4C; respectively). While the
 1953 diameters of the frontal ommatidia were positively related to body size in diurnal bees, these
 1954 ommatidia did not significantly vary according to body size in crepuscular bees (Figure 4B).
 1955 Although the diameters of ventral ommatidia were positively related to body size both in diurnal
 1956 and crepuscular bees, the inclination of the line is significantly lower in crepuscular bees than
 1957 in diurnal bees (Figure 4C). This indicates that smaller crepuscular bees have larger frontal and
 1958 ventral ommatidia than expected for their body size.

1959 Ommatidial diameters significantly differ between distinct eye regions in crepuscular
 1960 bees of the Halictidae and the Colletidae family (Figure 4D; $\chi^2= 1427$, $df= 2$, $p<0.001$; $\chi^2= 295$,
 1961 $df= 2$, $p<0.001$, respectively). In both families, the diameter of the dorsal ommatidia is smaller
 1962 than that of the frontal and ventral ommatidia (Figure 4D; $p<0.001$ in both cases). The diameter
 1963 of frontal and ventral ommatidia did not differ in both Halictidae and Colletidae crepuscular
 1964 bees (Figure 4d, $p=0.21$ and $p=0.46$, respectively).



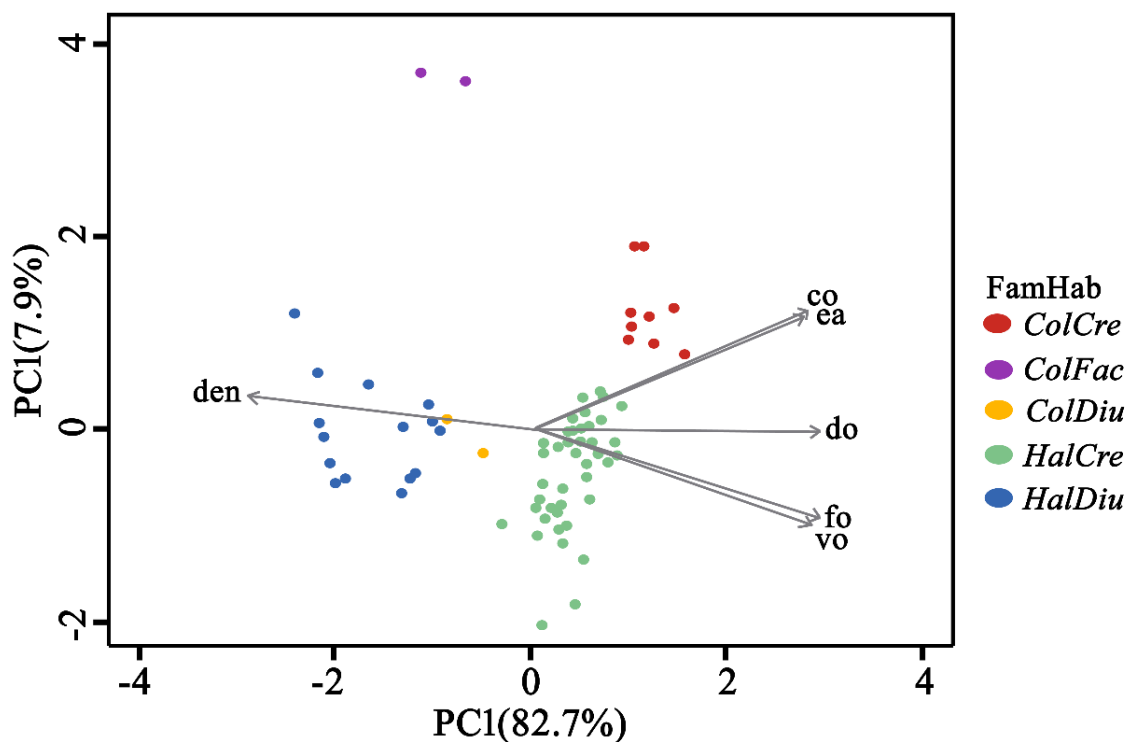
1965

1966 **Figure 4.** Plots from LMM testing the effects of the temporal patterns and the intertegular
 1967 distance (body size) on the diameter of dorsal (A), frontal (B) and ventral (C) eye ommatidia.
 1968 Bold lines indicate crepuscular bees and dotted lines indicate diurnal bees. Red dots: Colletidae
 1969 crepuscular bees; Orange dots: Colletidae diurnal bees; Green dots: Halictidae crepuscular bees;
 1970 Blue dots: Halictidae diurnal bees. The shaded areas correspond to the confidence intervals. D)
 1971 Box plots of ommatidial diameter in different eye regions of crepuscular bees of the Halictidae
 1972 (in green) and Colletidae (in red) families. FamHab: family and habit. TemPat: temporal
 1973 patterns. Asterisk (*) indicates statistical difference in the linear model.

1974 PCA analyzes returned two principal components that explained about 91% (PC1:
 1975 82.73%; PC2: 7.98%) of the total variation of optical features measured in crepuscular and
 1976 diurnal bees of families Colletidae and Halictidae (Figure 5). In this analysis, we also included
 1977 data of two facultative crepuscular individuals (*Caupolicana yarrowi*) from family Colletidae
 1978 (Figure 5, purple dots). Five out of the six variables (Figure 5; co, ea, do, fo, vo) were positively
 1979 correlated to PC1 and presented factor load values higher than 0.88. Just the ommatidial density

1980 (Figure 5; den) is inversely, but also strongly correlated to PC1, presenting load factor value of
 1981 -0.92. All six variables were much less correlated to PC2 and presented factor loading values
 1982 smaller than 0.4. Whereas the diameter of the central ocellus (co) and the eye area (ea) and
 1983 ommatidial density were positively correlated, the diameters of frontal (fo) and ventral (vo)
 1984 ommatidia were negatively correlated to PC2 (Figure 5).

1985 Bees with different temporal patterns and families were clearly distributed in different
 1986 positions of the PCA (Figure 5). The distribution of diurnal bees of Halictidae and Colletidae
 1987 family in the left side of the PCA were mainly associated to their higher ommatidial densities.
 1988 Crepuscular bees are mostly grouped in the right side of the PCA. The crepuscular Halictidae
 1989 bees were more associated to larger ommatidia of all regions of the eye, while the crepuscular
 1990 Colletidae are more associated to their large eyes and ocelli. The crepuscular facultative
 1991 Colletidae bees are grouped in the right side and upwards of the PCA, by the PC1 these bees
 1992 have great ommatidial density, and small ommatidia. By PC2 they are more associated to large
 1993 eye and ocelli, and like PC1 with small ommatidia.

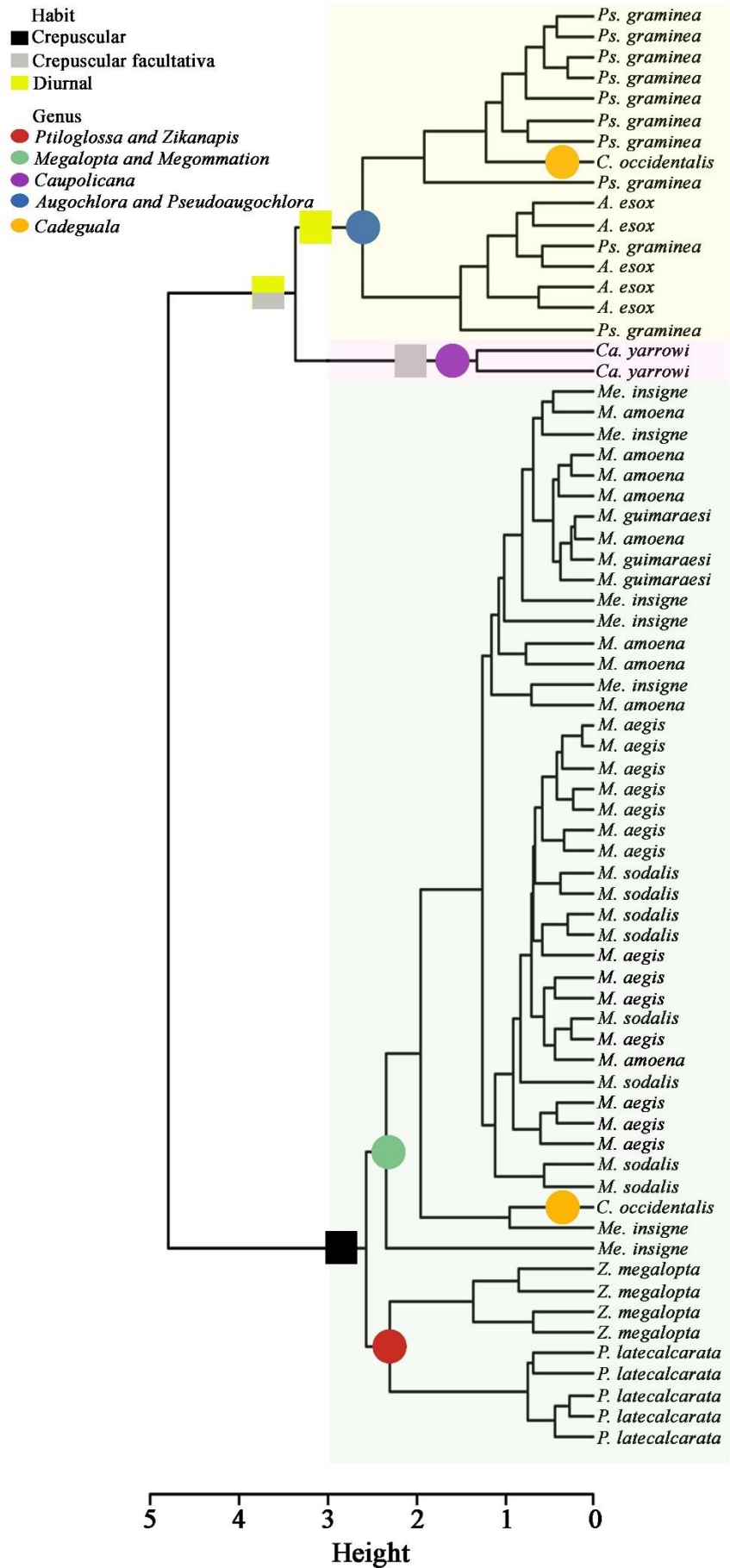


1994

1995 **Figure 5.** Analysis of main components of the morphological characters of the visual system
 1996 of bees which belong to different temporal patterns. Abbreviations: ColDiu = diurnal
 1997 Colletidae, ColFac = crepuscular facultative Colletidae, ColCre = crepuscular Colletidae,

1998 HalDiu = diurnal Halictidae and HalCre = crepuscular Halictidae. co = diameter of central
1999 ocellus, ea = eye area, den = ommatidial density, do = dorsal ommatidia, fo = frontal ommatidia,
2000 and vo = ventral ommatidia.

2001 Cluster analysis (Figure 6) shows that the temporal patterns was more important to
2002 group the species than the families to which they belong. In other words, we found more
2003 similarity in the visual system of bees that present the same temporal habitat than between bees
2004 that belong to the same family. The analysis revealed three well separated groups: diurnal,
2005 crepuscular and facultative crepuscular bees (Figure 6). We also can see the crepuscular
2006 facultative bees from Colletidae family have slightly more similarity with the diurnal bees than
2007 with the truly crepuscular. Individuals of a same genus or species were clearly grouped together
2008 in one of the major three groups, apart for one single Colletidae diurnal individual (*Cadeguala*
2009 *occidentalis*) that was unexpectedly grouped with Halictidae crepuscular bees.



2011 **Figure 6.** Dendrogram obtained by Cluster analysis, using the mean Euclidean distance for
 2012 grouping the species based in six visual attributes: diameter of the ocellus, eye area, ommatidial
 2013 density, diameters of the dorsal, frontal e ventral ommatidia. Square: temporal patterns, Circle:
 2014 genus. Black square: crepuscular habit; Grey square: crepuscular facultative habit; Yellow
 2015 square: diurnal habit. Red circle: *Ptiloglossa* and *Zikanapis* genus; Green circle: *Megalopta* and
 2016 *Megommation* bees; Purple circle: *Caupolicana* genus; Blue circle: *Augochlora* and
 2017 *Pseudaugochlora* genus; Orange circle: *Cadeguala* genus.

2018 **4. DISCUSSION**

2019 Here we show that the architecture of the visual organs of Halictidae and Colletidae bees
 2020 is related to both body size and temporal pattern. Crepuscular bees clearly have larger visual
 2021 organs than diurnal ones. While a direct positive relation exists both in diurnal and crepuscular
 2022 bees between the body size and the sizes of the ocelli, compound eyes, dorsal and ventral eye
 2023 ommatidia, two visual attributes did not directly relate to the body size in crepuscular bees: the
 2024 ommatidial density and size of frontal ommatidia. We found that these two specific eye
 2025 attributes are rather equivalent in small and large crepuscular bees. The values found in the
 2026 ommatidial density of crepuscular bees probably reflecting a conserved threshold between
 2027 visual sensitivity and resolution, which is probably an important adaptation to forage in low
 2028 light environments. Interestingly, multivariate analysis of six distinct morphometric attributes
 2029 of the visual organs revealed that crepuscular bees of distinct families share more similarity in
 2030 their visual organs than crepuscular and diurnal bees of a same family.

2031 **Body size and the visual organs of bees**

2032 Taking into account that the studied colletid bees are clearly bigger than Halictidae bees,
 2033 and also that different species within a family vary in size, we asked if and how body size is
 2034 related to the structure of visual organs in crepuscular and diurnal bees. We found that the
 2035 diameter of the central ocellus, as well as the area of the compound eye, is directly proportional
 2036 to body size in both crepuscular and diurnal bees. However, both ocelli and eye sizes are
 2037 consistently higher in crepuscular than in diurnal bees, a result that was expected considering
 2038 similar findings of studies comparing the size of visual structures of the crepuscular Halictidae
 2039 bee *Megalopta genalis* to the ones of other diurnal species (Jander and Jander, 2002; Kelber et
 2040 al., 2006). While *M. genalis* has been the major model of crepuscular bee studied at the
 2041 morphophysiological level (Warrant et al., 2004; Greiner et al., 2004ab; Frederiksen et al.,

2042 2008), Colletidae crepuscular bees have so far been poorly studied in terms of their visual
2043 morphology. Our data thus reinforces the notion that crepuscular bees of different taxonomic
2044 groups have larger and consequently more sensitive visual structures than their diurnal
2045 representatives.

2046 Body size appeared to be negatively related to the ommatidial density of diurnal bees,
2047 whereas in crepuscular bees, ommatidial density values are similar regardless of body size.
2048 Besides that, Halictidae and Colletidae crepuscular bees have lower ommatidial density than
2049 their respective diurnal relatives. These results confirm that the eyes of crepuscular bees invest
2050 in light sensitivity, but are limited in spatial resolution when compared to diurnal bees, like
2051 previously described in other insect groups (Land 1997; Cronin et al., 2014; Yilmaz et al.,
2052 2014). The eyes of crepuscular bees, especially the smaller ones, probably operate at the limit
2053 between detectability and acuity. Larger ommatidial diameters would be necessary to increase
2054 sensitivity to light, but consequently, this would also increase the interommatidial angles,
2055 reducing visual acuity to a level that would not allow reliable imaging (Snyder 1977; Land
2056 1997ab). On the other hand, improving visual acuity by enhancing ommatidial density, would
2057 probably lead to insufficient number of photons absorbed by the photoreceptors, resulting in
2058 increased visual-noise that impairs the formation of a reliable image (Snyder 1977; Land
2059 1997ab; Warrant 2017). Previous studies suggest that the eyes of *Megalopta* bees indeed
2060 operate within their limits of visual sensibility (Warrant 1999; Jander and Jander, 2002;
2061 Frederiksen et al., 2008; Jones et al., 2020). Our results extend this hypothesis also to other
2062 crepuscular bees, such as *Megommation*, *Ptiloglossa* and *Zikanapis*.

2063 **Eye-region specializations in crepuscular bees**

2064 Since different regions of the bee compound eye display distinct functions and thus vary
2065 in their visual properties (Lehrer 1998; Jander and Jander 2002; Warrant et al., 2004; Greiner
2066 et al., 2004b), we evaluated how the dorsal, frontal, and ventral eye ommatidia vary in
2067 crepuscular and diurnal bees. As expected, we found that ommatidia from these three eye
2068 regions are larger in crepuscular bees than in diurnal bees. However, while the diameter of
2069 ommatidia in the frontal eye region is directly proportional to the body size of diurnal bees,
2070 they tend to present equivalent values independent of the crepuscular bee sizes. For example,
2071 Halictidae bees (*M. amoena*) with 2 mm of body size, have the same frontal ommatidia diameter
2072 as Colletidae bees (*Z. megalopta*) with 5.2 mm of body size. In general, we found that

2073 crepuscular bees of distinct sizes appear to have conserved large frontal ommatidia, suggesting
2074 a pressure of the low light environment to select bees with enlarged frontal ommatidia
2075 independent of their body sizes. In this case, the evolutionary changes to occupy the crepuscular
2076 niche is related to the ecological functions e.g., like looking for flowers, finding the nest
2077 (described below), and allowed by morphological and probably physiological traits.

2078 We found that frontal and ventral eye regions of crepuscular bees present larger
2079 ommatidia. In diurnal bees, the frontal and ventral region also has a large diameter (Jander and
2080 Jander, 2002; Warrant et al., 2004; Streinzer et al., 2016). In general, in diurnal bee frontal and
2081 ventral areas are important in landscape processing, flower location and diverse other types of
2082 ecological interactions involving vision (Lehrer 1998; Lehrer 1990). In addition, these eye
2083 regions process visual patterns on the sides and ground, respectively (Giger and Srinivasan,
2084 1997). In crepuscular bees, we know little about the functions of these regions of the eyes. The
2085 crepuscular bee *M. genalis* uses the frontal-ventral part of the eye to find the nest entrance
2086 (Warrant et al., 2004). While *Megalopta* bees build their nest in the dead wood located one
2087 meter above the ground (Wscilo et al, 2004; Warrant et al., 2004), *Megommation insignes* and
2088 the Colletidae species studied here excavate its nests in the soil and mark its entrance with an
2089 erect soil turret (Michener and Lange 1958; Janzen 1968; Rozen 1984; Sarzetti et al., 2014). In
2090 both these frameworks, the visual fields captured by frontal and ventral ommatidia are,
2091 probably, important for nest location by crepuscular bees, among others task, such as diurnal
2092 bees. It appears that larger diameters of frontal-ventral ommatidia were selected throughout
2093 evolutionary history to optimize photon capture and enable crepuscular bees to perform
2094 different visual tasks in low light environments.

2095 In the eyes of crepuscular bees, the diameters of the dorsal ommatidial are smaller than
2096 the frontal and ventral ommatidial (Figure 4d), as already described for *M. amoena* and *M.*
2097 *genalis* (Jones, et al., 2020), and for diurnal bees (Jander and Jander, 2002; Warrant et al., 2004;
2098 Streinzer et al., 2016). While foraging, *Megalopta genalis* use the dorsal visual field to learn
2099 and memorized the foliage patterns created by the canopy against the brighter night sky (Chaib
2100 et al., 2021). In addition, the only natural light source in the dim-light environment (like a day)
2101 is irradiated from celestial bodies, whose photons penetrate tropical forests through the canopy
2102 of trees (Warrant et al., 2020). Therefore, the dorsal part of the eyes of crepuscular bees is likely
2103 to receive more photons of light than other eye regions. In this context, the evolutionary
2104 pressures to select light-sensitive ommatidia have probably been lower in the dorsal than in the

2105 frontal and ventral eye regions. Here we show that this ommatidial trait of the dorsal eye region
 2106 is conserved in Halictidae and Colletidae crepuscular bees. However, due to their larger body
 2107 size, Colletidae have larger dorsal ommatidia than Halictidae crepuscular bees.

2108 **The visual structure signature of crepuscular bees**

2109 When we analyzed how the set of six optical parameters measured in our study grouped
 2110 individuals in a PCA, we clearly observed that the visual variables grouping crepuscular and
 2111 diurnal bees from different families were related to increased light sensitivity and increased
 2112 visual acuity, respectively. Furthermore, Halictidae and Colletidae crepuscular bees were
 2113 spatially separated, which was mainly related to the bigger size of the visual structures in
 2114 Colletidae. Although the body size was not included as a variable in the PCA, bees with larger
 2115 body size tend to have larger visual structures. Colletidae crepuscular bees that are bigger also
 2116 have larger eyes, ocellus, dorsal ommatidia than Halictidae crepuscular bees. Interestingly, the
 2117 visual features of facultative crepuscular bees clearly separate them in the PCA from the diurnal
 2118 or crepuscular bees. Although according to Weislo and Tierney (2009) facultative crepuscular
 2119 bees do not have external features of the visual system that are associated with an ability to
 2120 forage under dim-light conditions, we showed, according to the PCA, that facultative
 2121 crepuscular bees have large eyes and ocellus, like the Colletidae crepuscular bees, and smaller
 2122 diameter of ommatidia and high ommatidial density, like diurnal bees.

2123 To better understand how similar or different were the visual structures of the 11 bee
 2124 species analyzed in our study, we performed a cluster analysis and found that the facultative
 2125 crepuscular bees shared the same node with diurnal bees. This node was then divided in two
 2126 other clusters: one grouped all diurnal Halictidae bees; another grouped the two specimens of
 2127 the *Caupolicana yarrowi* crepuscular facultative bees. These clusters demonstrate that although
 2128 the visual system of this crepuscular facultative bee is more similar to the ones of diurnal than
 2129 crepuscular bees, it also presents morphological features that are different from those of diurnal
 2130 bees, as evidenced in the PCA. In Arizona, the foraging activity of a female *Ca. yarrowi* was
 2131 observed from the nest site on an overcast morning (Linsley and Cazier, 1970). These bees out
 2132 of their nest to collect floral resources between 4:56 h to 9:16 h (Linsley and Cazier, 1970). In
 2133 the evening *Ca. yarrowi* collect pollen between 17:50 h to 19:13 h (sunset ~18:51 h) (Linsley
 2134 and Cazier, 1963). Although *Ca. yarrowi* begin foraging before sunrise or extend their flight
 2135 activity after sunset, they have plasticity to forage after sunrise and before sunset. Perhaps the

2136 similarity between the visual system of the crepuscular facultative bees in relation to the
2137 crepuscular and diurnal specimens reflects their plasticity for forage in both temporal niches.

2138 The node shared by strictly crepuscular bees was divided in two clusters: the first
2139 grouped the Colletidae bees *Zikanapis* and *Ptiloglossa*; the second grouped Halictidae bees
2140 *Megalopta* and *Megommation*. Therefore, the visual organs of distinct species of crepuscular
2141 bees of a same family share more similarity than the ones of crepuscular species belonging to
2142 different families. Cluster analysis also clearly grouped the species of Halictidae diurnal bees.
2143 All in all, our study indicates that the temporal habit exerts greater evolutionary pressures than
2144 phylogenetic relationships on the visual system of bees.

2145 5. REFERENCES

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2307

CAPÍTULO IV

2308

SPECTRAL SENSITIVITY OF THE POSITIVE PHOTOTAXIS IN CREPUSCULAR

2309

BEEES

2310

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2318

2319 **ABSTRACT**

2320 Phototaxis is an innate orientation of organisms towards light, which can be either attraction
2321 (positive) or repulsion (negative). Bees have positive phototaxis. This behavior mediates the
2322 orientation towards the hive entrance, as well as escape and take-off flight responses. Diurnal
2323 bees are attracted by UV, blue and green light, among these light stimuli only blue light is labile.
2324 Also, their phototaxy response is stronger at higher light intensities. Crepuscular bees forage at
2325 twilight, when light intensity is dimmer than the day, and the solar irradiance spectrum also is
2326 different from day. The phototaxis behavior of crepuscular bees, however, has not yet been
2327 described. So, the aim of this study was to understand how phototactic behavior of crepuscular
2328 bees is modulated by light. Positive phototaxis tests were performed in a dark circular arena
2329 with 18 females of *Megalopta aegis* (Halictidae). The circular arena presented three pairs of the
2330 monochromatic LEDs: UV 350 nm, blue 440 nm, and green 525 nm with six light intensities
2331 each (6.0, 3.0, 1.5, 0.8, 0.4 and 0.2 $\mu\text{W}/\text{cm}^2/\text{nm}$). Each LED was positioned in front of another
2332 one with the same wavelength and light intensity. The individual bee was subjected to the
2333 sequence of lights of these three wavelengths and six light intensities. Thereafter, we evaluated
2334 the number of responses to light, time, length, instantaneous speed, and deviation angle by bees
2335 to reach each light. Unlike what is described for diurnal bees, *M. aegis* is not always attracted
2336 to light stimuli presented in the dark. Reduced attraction to light might have evolved in
2337 crepuscular bees as an adaptive trait related to navigation under low light. When *M. aegis*
2338 responded to light, in all metrics evaluated, they showed stronger phototaxis and/or better
2339 orientation towards UV than blue or green lights, and similar response to blue and green lights.
2340 This result suggests that although all photoreceptors contribute to phototaxis behavior, the S
2341 photoreceptor type, which absorbs light in the UV region, has a higher contribution to this
2342 behavior. A possible reason for the stronger effect of UV light in the phototactic orientation of
2343 *M. aegis* might be that more S receptors are connected to the visuomotor neural tracts involved
2344 in phototaxis.

2345

2346 **KEYWORDS:** phototaxis, innate behavior, light spectra, vision, nocturnal bees, *Megalopta*.

2347 1. INTRODUCTION

2348 Phototaxis, a locomotor response towards (positive phototaxis) or away from (negative
2349 phototaxis) light sources, is a well-characterized behavior in insects (Jander, 1963). Typically
2350 understood as innate and stereotyped (Nouvian & Galizia, 2020), this behavior is mainly
2351 influenced by light intensity and wavelength (Menzel and Greggers, 1985). Bees, in general,
2352 present positive phototaxis, which seems to mediate orientation towards the hive entrance, as
2353 well as escape and take-off flight responses (Bertholf, 1931; Labhart, 1974; Kaiser et al., 1977;
2354 Menzel and Greggers, 1985; Erber and Scheiner, 2006; Nouvin and Galizia, 2020). Although
2355 this behavior was identified and characterized by different studies in bees, and especially in
2356 *Apis mellifera* Linnaeus, 1758, its function and physiological bases are not fully understood.

2357 The attraction of bees towards light is mediated by both the compound eyes and the
2358 three simple-lens eyes, the ocelli (Vieira, 2018). Three photoreceptor types can be found in the
2359 compound eye of bees, with absorption peaks at UV 344 nm (S type), blue 436 nm (M type),
2360 and green 544 nm (L type) (Peitsch et al., 1992). In ocelli there are two types of photoreceptors,
2361 one sensitive to UV light (~345 nm) and the other to green one (~500 nm) (Ribi et al., 2011).
2362 Studies have demonstrated that honeybees (*A. mellifera*) respond to UV, blue and green lights,
2363 suggesting that all photoreceptor types are involved in their phototactic behavior (Kaiser et al.,
2364 1977; Menzel and Greggers, 1985). Although phototaxis apparently implicates different
2365 photoreceptors, it has been suggested that bees are probably color blind during this behavior
2366 (Menzel and Greggers, 1985), but further behavioral and psychophysical studies are necessary
2367 to understand if color processing is indeed absent in distinct contexts of the bee's phototactic
2368 response.

2369 Honeybees showed stronger phototaxis to ultraviolet, followed by green, and finally,
2370 blue light, when these stimuli were presented in equivalent physical intensity (Kaiser et al.,
2371 1977; Nouvian and Galizia et al., 2020). In addition, bees have stronger phototactic responses
2372 as the intensity of the light stimulus increases (Kaiser et al., 1977; Menzel and Greggers, 1985;
2373 Erber et al., 2006; Scheiner et al., 2014). Interestingly, only phototactic responses to blue light,
2374 but not to UV or green, were found to be labile, presenting some level of experience-dependent
2375 plasticity (Marchar et al., 2019; Nouvian and Galizia, 2020).

2376 Unlike diurnal bees, which forage at high light intensities, some bees search for floral
2377 resources only when the solar irradiance is very low (Warrant et al., 2004; Kelber et al., 2006;

2378 Liporoni et al., 2020). These are known as crepuscular bees, and their period of activity is
2379 mainly concentrated in the twilight. Kelber et al., (2006) suggested that crepuscular bees use
2380 phototaxis to find their way back to their nests. The same authors also proposed that crepuscular
2381 bees may use decreasing light intensities in the evening and increasing light intensities in the
2382 morning as cues to stop foraging activity. None of these hypotheses, however, were so far tested
2383 in controlled conditions. For forage in low light conditions, these bees have some adaptations
2384 in their visual system that increase light sensitivity, such as large ocelli, eyes, and facets, wide
2385 rhabdom diameter, and high contrast gain in photoreceptors (Kerfoot 1967; Jander and Jander,
2386 2002; Warrant et al., 2004; Greiner et al., 2004ab; Frederiksen et al., 2008).

2387 During the twilight, light levels are many orders of magnitude dimmer than the day
2388 (Theobald et al., 2007; O'Carroll and Warrant, 2017). Furthermore, the spectrum of twilight
2389 solar irradiance is different from the day one, with a blue peak centered at approximately 450
2390 nm (Cronin et al., 2014; Palmer and Johnsen, 2014). Considering that crepuscular bees navigate
2391 when solar radiation intensity and spectrum change very quickly, and also that their visual
2392 systems present distinct adaptations to increase light sensitivity (Warrant and Dacke, 2016), we
2393 aimed at studying how their phototactic behavior is modulated by light. More precisely, we
2394 performed orientation tests with different monochromatic light stimuli to uncover the spectral
2395 sensitivity of the phototactic behavior in the crepuscular bees *Megalopta aegis* (Vachal, 1904)
2396 (Halictidae: Augochlorini). We also compared the phototactic orientation of these crepuscular
2397 bees when a same light stimulus was presented in distinct intensities, ranging from low values
2398 equivalent to those of the solar irradiance during dawn/dusk until high values like the ones of
2399 day light. Our study shows that both light wavelength and intensity influence the phototactic
2400 behavior of crepuscular bees. Moreover, we found clear differences in the way light modulates
2401 phototaxis in crepuscular and diurnal bees.

2402

2403 **2. MATERIAL AND METHODS**

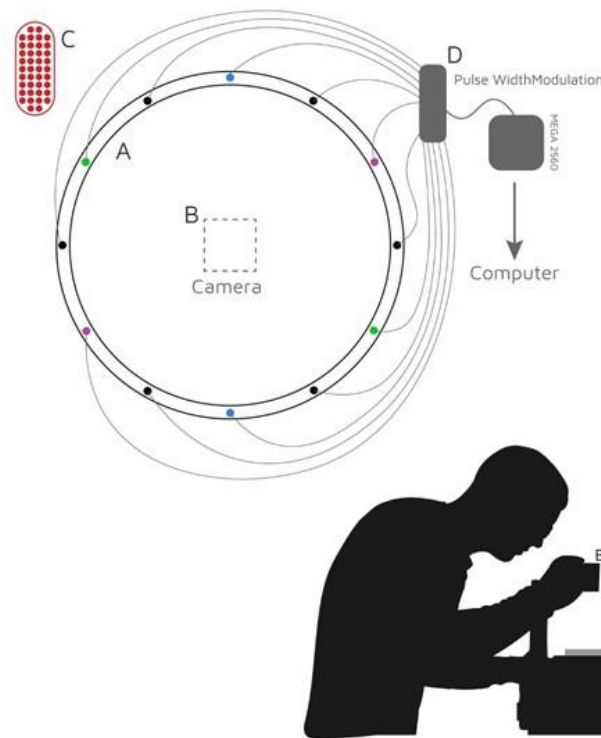
2404 **2.1 Bees**

2405 Bees of *Megalopta* genus (Halictidae: Augochlorini) are described as crepuscular
2406 (Wscilo and Tierney, 2009; Gonçalves 2016). We chose *Megalopta aegis*, a tropical crepuscular
2407 sweat bee, as a model to analyze phototactic orientation and its physiological bases. This bee
2408 species visits the flowers both at dawn and dusk (Siqueira et al. 2018; Araujo et al., 2020).

2409 *Megalopta aegis* female individuals were collected with light traps in Parque Estadual
2410 do Rio Preto (São Gonçalo do Rio Preto, MG) during dusk (17:30 – 19:00) and dawn (4:00 –
2411 6:00). After collection, each bee was individually placed in a plastic tube (diameter = 1 cm)
2412 with small holes all over its length (length = 5 cm), and then taken to the laboratory (LAFISC,
2413 ICB-UFMG, Belo Horizonte, MG). The plastic tubes with the bees were kept inside a dark
2414 chamber with monitored temperature (25-28 °C), and experiments were conducted within the
2415 following five days. Crepuscular bees remain inside their nests throughout the day (Warrant et
2416 al., 2004; Kelber et al., 2006), in an environment likely to be poorly lit. So, in the lab, we kept
2417 the animals in the darkness. The bees were fed with water and sugar solution (30%) once at
2418 dusk (~17:30 h). We studied the positive phototaxis in a total of 18 female crepuscular bees.

2419 **2.2 Experimental setup**

2420 We studied the positive phototaxis of individual *Megalopta* bees by recording
2421 orientation trajectories in a circular arena developed by Erber et al. (2006) to analyze the
2422 phototactic behavior of *Apis mellifera*. This experimental arena was made of opaque black
2423 acrylic sheets and had an internal diameter of 35 cm, with a circular lateral wall of 1 cm in
2424 height (Figure 1). A transparent acrylic cover allowed the recording of the bee orientation by
2425 an infrared camera (model SJCAM SJ4000-30 FPS, 1080 P with IR-filter removed) placed
2426 above (~ 35 cm high) the center of the arena (Figure 1). An infrared LED spotlight was placed
2427 around the arena to allow recording bees' trajectories in the dark with reasonable spatial
2428 resolution. Infrared light was highly reflected by the *Megalopta* bee body, thus enhancing bee
2429 visibility on the video recordings. Monochromatic LEDs used as visual stimuli (see next
2430 section) were disposed in 12 holes (5 mm in diameter) along the circular wall of the arena, so
2431 that each stimulus was positioned at an angle of 30° to the center of arena. All experiments were
2432 performed in a dark room to avoid any interference from others visual stimuli.



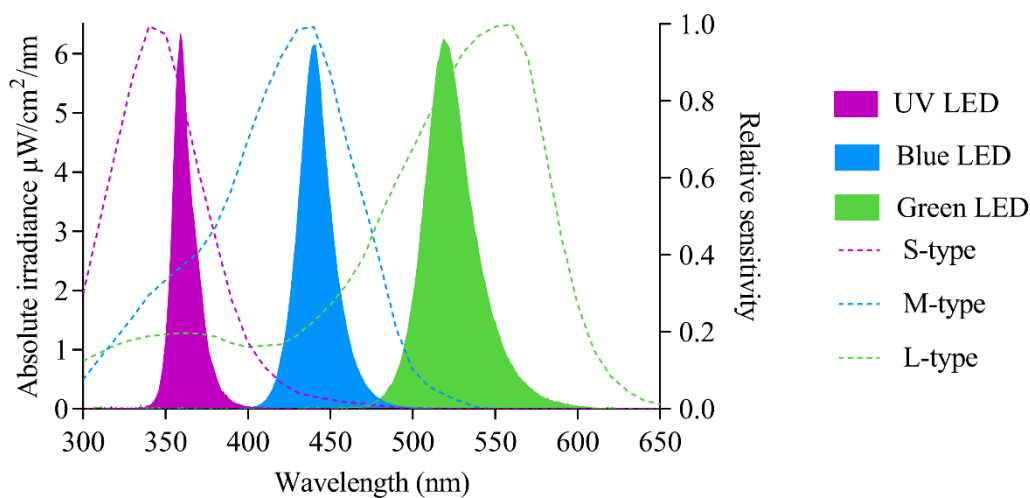
2433

2434 **Figure 1.** Experimental setup used for recording the phototactic trajectories of *Megalopta aegis*
 2435 individual bees. A) Circular arena used to present monochromatic stimuli to the bee. Purple
 2436 circle: pair of UV LEDs (355 nm), blue circle: pair of blue LEDs (440 nm), green circle: pair
 2437 of green LEDs (525 nm), black circle: other positions in which LEDs could be presented. Pairs
 2438 of a same stimulus always had a distance of 35 cm between stimuli. B) Infrared camera
 2439 positioned above the center of the arena. C) Infrared spotlight. D) Arduino[®] (MEGA 2560) was
 2440 used to modulate the presentation of each monochromatic stimulus in specific values of
 2441 irradiance. The order of presentation of pairs of stimuli with distinct wavelengths and intensities
 2442 was also controlled by Arduino, using a custom-made Python code.

2443 2.3 Visual stimuli

2444 Light-emitting diodes (LED) of different wavelengths and intensities were frontally
 2445 presented to the walking bee within the circular experimental arena (Figure 1). Three
 2446 monochromatic LEDs with emission peaks in 355 nm (Roithner Lasertechnik; XSL-355-5E-
 2447 R6), 440 nm (Roithner Lasertechnik, LED440-6-30) and 525 nm (Roithner Lasertechnik; B5-
 2448 433-B525) were used to produce visual stimuli (Figure 2). The emission peaks of these LEDs
 2449 correspond to the absorption peaks of the three photoreceptor types (Figure 2) described in
 2450 honeybees and most of the Hymenoptera species studied so far (Peitsch et al., 1992). A

2451 spectrophotometer (USB2000+UV-VIS-ES, Ocean Optics, Dunedin, FL, USA) radiometrically
 2452 calibrated by means of a deuterium/tungsten light source (DH-2000-BAL, 220–1050 nm, Ocean
 2453 Optics) was used to measure and adjust the absolute irradiance of the monochromatic stimuli.
 2454 Each LED was connected to a pulse width modulation (PWM) signal output port in Arduino[®]
 2455 (MEGA 2560), thus allowing to control its active cycle and to modulate its absolute irradiance.
 2456 Absolute irradiance from 300 to 800 nm was measured using an optical fiber (QP600-2-UV-
 2457 VIS, Ocean Optics) coupled to a cosine corrector with Spectralon diffusing material (CC-3-
 2458 UV-S, Ocean Optics). The software SpectraSuite (Ocean Optics) was used for acquisition and
 2459 analysis of spectral curves. We used the following logarithmic range of relative light intensity:
 2460 100%, 50%, 25%, 12.5%, 6.25% and 3.125%, corresponding to absolute irradiance values of
 2461 $6.0 \mu\text{W}/\text{cm}^2/\text{nm}$, $3.0 \mu\text{W}/\text{cm}^2/\text{nm}$, $1.5 \mu\text{W}/\text{cm}^2/\text{nm}$, $0.8 \mu\text{W}/\text{cm}^2/\text{nm}$, $0.4 \mu\text{W}/\text{cm}^2/\text{nm}$ and 0.2
 2462 $\mu\text{W}/\text{cm}^2/\text{nm}$, respectively. The order of presentation of stimuli with distinct wavelengths and
 2463 intensities was controlled by Arduino[®] through a custom-made software developed in Python
 2464 (version 3.0).



2465

2466 **Figure 2.** Absolute irradiance of each monochromatic LED at the highest intensity tested
 2467 (100%) and the absorption spectrum of the three honeybee photoreceptor types. Solid peaks:
 2468 irradiance spectra of the monochromatic UV, blue and green LEDs. Dotted curves: relative
 2469 sensitivity of each photoreceptor type from 300 to 650 nm.

2470 2.4 Phototactic behavior

2471 *Megalopta aegis* individuals kept in our laboratory appeared to be active and reactive to
 2472 light only during crepuscular periods (data not shown). We thus decided to perform all

2473 behavioral assays at dusk, more precisely from 17:20 h to 18:20 h (maximum). The spectral
 2474 sensitivity of the positive phototaxis of 18 individual bees was analyzed by recording their
 2475 trajectories towards visual stimuli of 3 wavelengths (UV, blue or green), which were presented
 2476 in 6 distinct irradiance values. The sequence of presentation of these three wavelengths was
 2477 randomized between bees (e.g. UV-blue-green, blue-UV-green, green-UV-blue or any other
 2478 possible arrangement). Each monochromatic stimulus was tested in a sequence of six increasing
 2479 intensities, as performed by Erber and colleagues (2006) when testing honeybee phototaxis to
 2480 green light.

2481 After an individual bee was introduced into the arena, in complete darkness, we turned
 2482 on a UV stimulus and waited for the bee to respond. When the bee walked towards this light,
 2483 we switched it off for 5 minutes, and then started the series of phototaxis tests. Each of the 18
 2484 visual stimuli (3 wavelengths x 6 intensities) was presented until the bee reached it or for a
 2485 maximum duration of 30 seconds. We then turned off that stimulus, and turned on its equivalent
 2486 pair that was positioned at a distance of 35 cm, in the opposite side of the arena (Figure 1). This
 2487 switch of light presentation in two opposite equidistant positions was repeated for more three
 2488 times, thus allowing the recording of three subsequent phototactic orientation paths per
 2489 stimulus. A time-interval of 10 s in complete darkness was provided between presentation of
 2490 each intensity of a same light source. When the wavelength was changed, we applied a time-
 2491 interval of 30 s. The first path to reach each new stimulus was not considered in our analyses,
 2492 since the position from which the bee starts locomotion is not at a precise distance of 35 cm
 2493 from the light source, as for the next three paths recorded.

2494 **2.5 Image analysis**

2495 Video recordings were analyzed in MATLAB[®] (version R2019b), using the ZebTrack
 2496 extension (version 2.6.1) developed by Pinheiro-da-Silva et al., (2017) and complementary
 2497 customized codes. The Table 1 describes the six parameters we evaluated in the phototactic
 2498 paths of crepuscular bees towards distinct spectral stimuli.

2499 **Table 1.** Parameters extracted from video recordings of the locomotory activity of walking
 2500 *Megalopta aegis* towards monochromatic light stimuli presented in a dark circular arena.

Parameter	Description
Number of positive phototactic responses	Number of times the bee moved towards the light stimulus.

Time (s)	Time spent by the bee to reach the light stimulus.	
Length (cm)	Total length traveled by the bee to reach the light stimulus.	
Instantaneous speed (cm/s ²)	speed	Speed in every centimeter of the path towards light.
Deviation (degree/frame)	angle	Deviation angle, per frame, between the velocity vector and the vector from the bee to the center of the light source.

2501

2502 **2.6 Statistical analysis**

2503 General linear mixed model (GLMM) was used to test how light wavelength and
2504 intensity influenced the number of phototactic responses of *M. aegis*, assuming a Poisson data
2505 distribution. We considered the number of positive phototactic reactions as the response
2506 variable, light wavelength and intensity as two fixed effects (predictor variables), and the bee
2507 ID as a random effect. Linear Mixed Model analysis (LMM) was used to test how light
2508 wavelength and intensity affected the duration and the length of the phototactic paths of *M.*
2509 *aegis*. Time (s) and length (cm) were the response variables, whereas light wavelength and
2510 intensity were considered as fixed effects, and the bee ID as a random effect.

2511 Aiming at a deeper comprehension of the locomotory dynamics of *M. aegis* during
2512 phototactic orientation towards distinct spectral stimuli, we developed a LMM to evaluate the
2513 effects of light wavelength, intensity and distance on the instantaneous speed (cm/s²) and the
2514 deviation angle (degree/frame) from a straight path. We considered the deviation angle
2515 (degree/frame) and the instantaneous speed (cm/s²) as the response variables. Light wavelength,
2516 intensity and distance were considered as fixed effect (predictor variables), and the bee ID as a
2517 random effect.

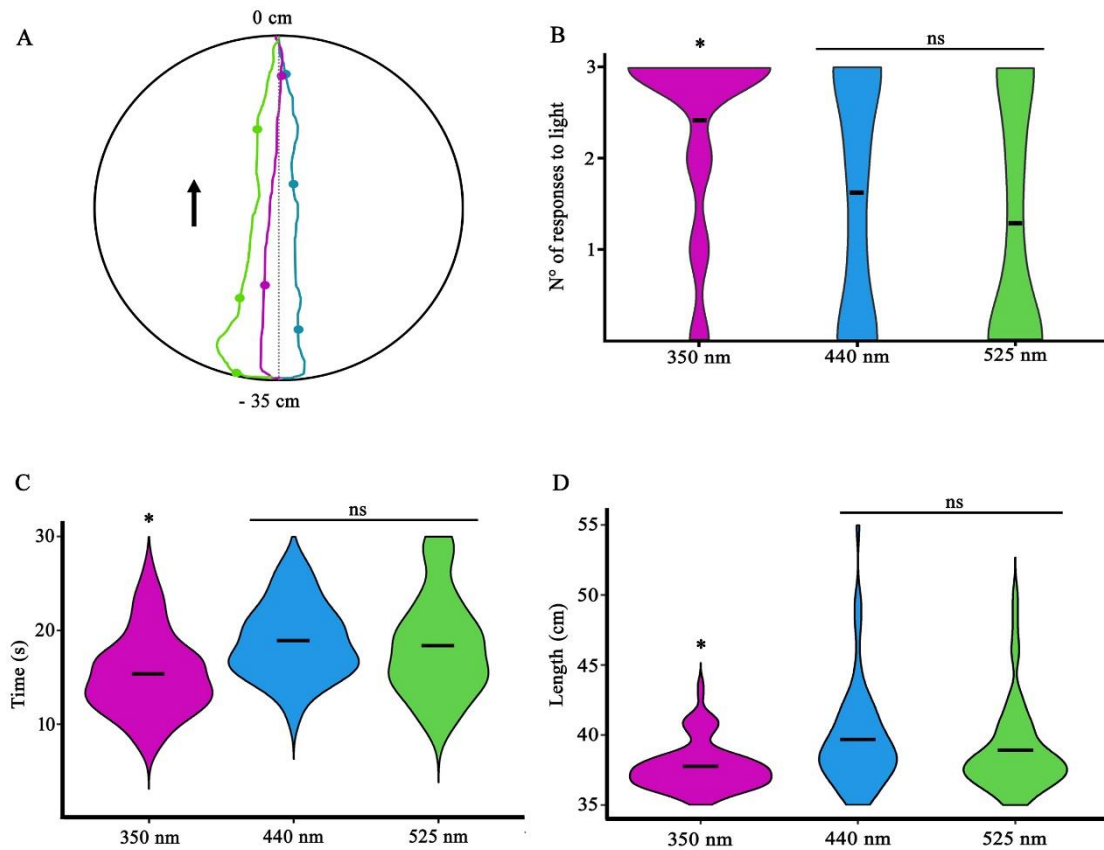
2518 All the analysis and graphic representations of data were carried out in the software R
2519 (R Core Team 2018). The packages lme4 (Bates et al., 2015) and multcomp (Hothorn et al.,
2520 2016) were used to develop LMM, GLMM and planned comparisons. Figures were produced
2521 using the ggplot2 package (Wickham, 2011).

2522

2523 **3. RESULTS**

2524 During phototactic tests performed in a dark circular arena (Figure 1), *Megalopta aegis*
 2525 bees were not always responsive to all presentations of the monochromatic stimuli (Figure 2).
 2526 Figure 3A shows examples of walking paths recorded in the circular arena (35 cm) for
 2527 presentations of a monochromatic UV (350 nm), blue (440 nm) or green (525 nm) light spot of
 2528 equivalent absolute irradiances. The number of positive phototactic responses of *M. aegis*
 2529 towards a stimulus was significantly influenced by light wavelength ($\chi^2_{\text{wavelength}}=885$, $df=2$,
 2530 $p<0.001$, Figure 3B), but not by light intensity or the interaction between light wavelength and
 2531 intensity (respectively, $\chi^2_{\text{intensity}}=5$, $df=5$, $p=0.38$, $\chi^2_{\text{wavelength*intensity}}=5$, $df=10$, $p=0.14$). Bees
 2532 reacted more frequently to UV than to blue or green lights ($p<0.001$, Figure 3B), whereas the
 2533 number of responses to blue and green lights was equivalent ($p=0.97$, Figure 3B).

2534 Both the duration and the length of the phototactic paths of *M. aegis* were also
 2535 significantly modulated only by light wavelength (respectively, $\chi^2_{\text{wavelength}}=58$, $df=2$, $p<0.001$
 2536 and $\chi^2_{\text{wavelength}}=26$, $df=2$, $p<0.001$), but not by light intensity ($\chi^2_{\text{intensity}}=6$, $df=5$, $p=0.24$ and
 2537 $\chi^2_{\text{intensity}}=8$, $df=5$, $p=0.12$, respectively) or the interaction between light wavelength and intensity
 2538 ($\chi^2_{\text{wavelength*intensity}}=11$, $df=10$, $p=0.35$ and $\chi^2_{\text{wavelength*intensity}}=11$, $df=10$, $p=0.34$, respectively).
 2539 Periods of time (Figure 3C) and lengths of phototactic paths (Figure 3D) were significantly
 2540 shorter to UV than to blue or green lights ($p<0.05$). Durations (Figure 3C) and lengths of the
 2541 phototactic paths (Figure 3D) towards blue and green light were equivalent ($p=0.81$, $p=0.16$,
 2542 respectively).



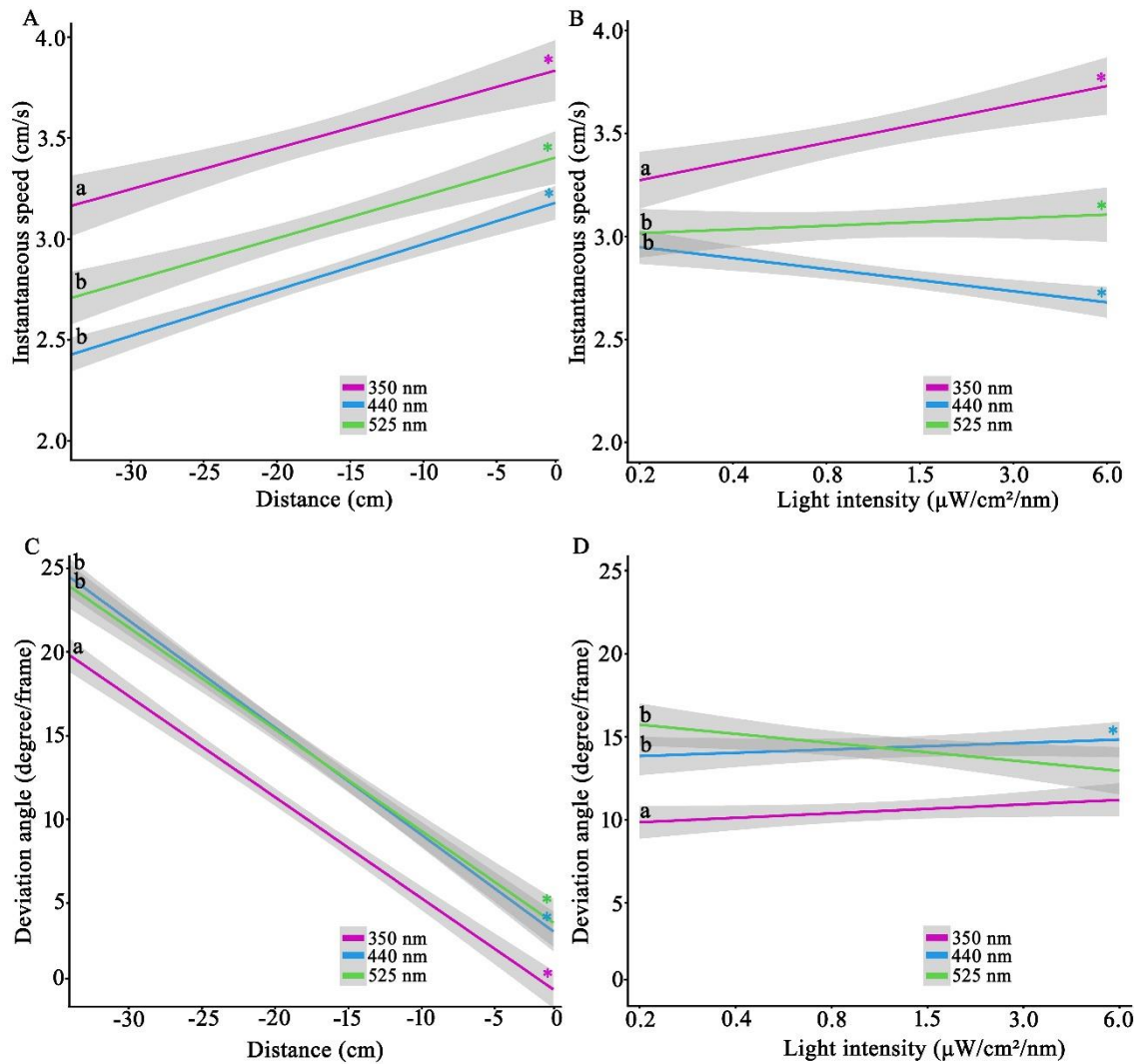
2543

2544 **Figure 3.** Phototactic paths of *M. aegis* recorded in a dark circular arena (35 cm) presenting
 2545 monochromatic UV, blue or green light stimuli. A) Examples of individual paths towards UV
 2546 (purple line), blue (blue line), and green (green line) lights of a same irradiance value (0.2
 2547 $\mu\text{W}/\text{cm}^2/\text{nm}$). The arrow indicates the direction of the bee's walking towards the light stimulus.
 2548 The dotted gray line indicates the direct path to the light stimulus. The dots indicate the position
 2549 of bee every 5 seconds from the starting time. We represent all three monochromatic stimuli at
 2550 a same position of the arena for a better comparison of the phototactic paths, but position and
 2551 sequence of stimuli presentation were randomized between trials and individuals. B – D) Violin
 2552 plots showing the effects of light wavelength (UV 350nm, blue 440nm or green 525nm) on the
 2553 following response variables: number of responses to light (B), time (C) and length (D) of the
 2554 phototactic response paths. The widths of violin plot regions represent the distribution, and the
 2555 black lines indicate the mean of values obtained in 18 animals. Asterisks indicate significant
 2556 differences ($p < 0.05$) between distinct wavelengths in GLMM (B) or LMM (C-D) analyses.
 2557 Non-significant differences are indicated by 'ns'.

2558 Although the average durations and lengths of phototactic paths (Figure 3) did not
 2559 appear to be significantly influenced by the intensity of a same light wavelength, further

2560 analysis of the instantaneous speed at different distances from the light stimulus (-35 to 0 cm)
 2561 uncovered significant effects of light distance, intensity and wavelength in bee's acceleration
 2562 dynamics ($\chi^2_{\text{distance*intensity*wavelength}} = 6454$, $df= 17$, $p<0.001$, Figure 4 A-B). The instantaneous
 2563 speed of bees significantly increased as the distance towards a light stimulus decreased
 2564 ($\chi^2_{\text{distance}}=347$, $df=8$, $p<0.001$, Figure 4A). Moreover, bees were faster when walking towards
 2565 UV than blue and green lights ($\chi^2_{\text{wavelength}}=557$, $df=14$, $p<0.001$, Figure 4A). Light intensity also
 2566 significantly influenced the instantaneous speed of bees ($\chi^2_{\text{intensity}}=155$, $df=20$, $p<0.001$),
 2567 however, this effect was different for distinct wavelengths (Figure 4B). As the intensity of UV
 2568 light increased, the instantaneous speed of *M. aegis* also increased in an irradiance-dependent
 2569 manner ($p\leq 0.001$ in two pairwise comparisons; Figure 4B). Similar, but much less pronounced
 2570 effect of light intensity was also found on the instantaneous speed of bees towards green stimuli
 2571 of increasing irradiance ($p<0.01$ in two pairwise comparisons; Figure 4B). Contrarily to the
 2572 other two stimuli, the mean instantaneous speed of bees significantly decreased as the intensity
 2573 of blue light increased ($p<0.001$ in five pairwise comparisons; Figure 4B).

2574 As a measure of tortuosity, we analyzed the deviation angle between the velocity vector
 2575 of a walking bee and the vector pointing to the light spot position in each frame of a recorded
 2576 phototactic path. We found that the dynamics of this parameter is also significantly affected by
 2577 light distance, intensity, and wavelength ($\chi^2_{\text{distance*intensity*wavelength}}= 35$, $df= 17$, $p=0.005$, Figure
 2578 4 C-D). The tortuosity of the phototactic paths of *M. aegis* decreased as the distance toward the
 2579 led also decreased ($\chi^2_{\text{distance}}=1095$, $df=8$, $p<0.001$, Figure 4C). Furthermore, the deviation angle
 2580 was smaller when bees walked towards UV than blue and green lights ($\chi^2_{\text{wavelength}}=51$, $df=20$,
 2581 $p=0.0001$, Figure 4C). Although light intensity appeared to influence the average deviation
 2582 angle of bees, further multiple comparisons revealed a single significant effect between 0.8 and
 2583 $6.0 \mu\text{W}/\text{cm}^2/\text{nm}$ intensities of blue light ($p=0.04$). We identified no significant effect of light
 2584 intensity on the tortuosity of phototactic paths towards UV or green lights (Figure 4D).



2585

2586 **Figure 4.** Acceleration and tortuosity dynamics of the phototactic walking paths towards UV,
 2587 blue and green monochromatic stimuli presented in different absolute irradiance values. A)
 2588 Instantaneous speed of *M. aegis* at different distances (-35 to 0 cm) from the light stimulus. B)
 2589 Instantaneous speed of bees during phototactic orientation towards increasing light intensities.
 2590 C) Derivation angle between the velocity vector and the straightest vector pointing to the LED
 2591 at different distances (-35 to 0 cm) from the light stimulus. D) Derivation angle of bees during
 2592 walking phototactic paths towards increasing light intensities. Purple line: 350 nm, blue line:
 2593 440 nm, green line: 525 nm. The shaded gray areas represent standard deviation from mean.
 2594 Different letters indicate statistical difference between wavelengths. Asterisks indicate
 2595 significant effect of distance (A and C) or light intensity (B and D) on each response variable.

2596 4. DISCUSSION

2597 Here we analyzed the visuomotor dynamics and spectral sensitivity of the positive
2598 phototaxis for the first time in a crepuscular bee species. Different from previous studies
2599 performed in diurnal bees (Bertholf 1931; Heintz 1959; Kaiser et al. 1977; Menzel and
2600 Greggers, 1985; Erber and Scheiner, 2006; Nouvin and Galizia, 2020), we found that
2601 *Megalopta aegis* is not always attracted to light stimuli presented in the dark. This absence of
2602 spontaneous phototaxis at times found in this crepuscular species, never before reported for
2603 *Apis* or *Bombus* species (Marchal et al., 2019; Novin and Galizia, 2020; Merling et al., 2020).
2604 Reduced attraction to light might have evolved in crepuscular bees as an adaptive trait related
2605 to navigation under low light, but further studies are still necessary to support this hypothesis.

2606 The frequency of phototactic responses of *M. aegis* is higher to UV than to blue or green
2607 lights, indicating a stronger contribution of shorter wavelengths in eliciting this behavior in
2608 crepuscular bees. Furthermore, all parameters evaluated in the phototactic paths of *M. aegis*
2609 (e.g. duration and length) indicate stronger phototaxis and/or better orientation towards UV than
2610 towards blue or green lights. As the crepuscular bee approached a light source of any
2611 wavelength, its instantaneous speed increased, and the tortuosity of the trajectory gradually
2612 decreased. However, speed was higher and tortuosity (deviation angle/frame) was lower to UV
2613 than to blue and green lights. These results point out to different contributions from the distinct
2614 types of photoreceptors to the phototactic behavior of *M. aegis*.

2615 Stronger attraction to UV over longer wavelength lights is also observed in many other
2616 insects like flies, moth and hemipterans (Green and Cosens, 1983; Gao et al., 2008; Yamaguchi
2617 and Heisenberg, 2011; Paris et al., 2016; Tokushima et al., 2016; Brehm et al., 2021), and in
2618 the honeybee *Apis mellifera* (Bertholf 1931, Heintz 1959, Labhart, 1974; Kaiser et al. 1977;
2619 Nouvin and Galizia, 2020). Besides that, most studies showed that honeybees are more attracted
2620 to green than to blue light presented in the dark (Bertholf, 1931; Heintz, 1959; Labhart, 1974;
2621 Vieira et al., 2018; Nouvin and Galizia, 2020). Some works, however, found no clear
2622 differences in the attraction of bees to blue and green lights (Kaiser et al., 1977; Menzel and
2623 Greggers, 1985). The main parameters here analyzed in the phototactic paths displayed by
2624 crepuscular bees presented no significant differences between blue and green lights. Thus, a
2625 clear overall pattern emerges from our data: *M. aegis* presents a stronger phototaxis to UV than
2626 to other wavelengths, while the attraction to blue and green lights is similar. This result suggests
2627 that the S photoreceptor type, which absorbs light in the UV region, has a higher contribution

2628 to the phototactic orientation of *M. aegis* than the M and L receptors that absorb lights in the
2629 blue and green regions, respectively.

2630 So far, the contribution of distinct photoreceptors to the phototactic behavior of diurnal
2631 social bees is not well elucidated. For the honeybee *A. mellifera*, Lahhart (1974) proposed that
2632 only the S and L receptor types were involved in phototaxis, since responses to blue were
2633 weaker than to UV and green. Contrarily, Kaiser and colleagues (1977) found no differences
2634 between responses of honeybees to blue and green, suggesting that all three photoreceptor types
2635 participate in the honeybee innate attraction to light. Menzel and Greggers (1985) performed a
2636 different experimental design to evaluate the natural phototaxis of free-flying honeybees that
2637 leave a feeding place and start to fly back to the hive. In such a different context, in which bees
2638 were adapted to sunlight, prior to the phototactic test performed in a rewarded darker chamber,
2639 these authors found similar attraction to UV, blue and green lights and suggested a balanced
2640 contribution of the three photoreceptor types to phototaxis. Our data also suggest the
2641 contribution of all three photoreceptor types to the phototaxis of crepuscular bees, but the S
2642 photoreceptor type appears to have a stronger role in this behavior. A possible reason for the
2643 stronger effect of UV light in the phototactic orientation of *M. aegis* might be that more S
2644 receptors are connected to the visuomotor neural tracts involved in phototaxis. In other bee
2645 behaviors, differences in the contributions of distinct photoreceptor types were already
2646 described. For example, bees only use S receptors to detect the polarized-light pattern in the
2647 sky (Lahhart, 1980), while L receptors are most important than the others to achromatic vision
2648 and motion-related tasks (Menzel, 1974; Skorupski and Chittka, 2010).

2649 Compared to *A. mellifera*, we found that *M. aegis* displayed shorter and slower
2650 phototactic paths to monochromatic green light presented in equivalent setup and experimental
2651 design (Erber and Sheiner, 2006). These behavioral differences between diurnal and
2652 crepuscular bees probably reflect the characteristics of their visual systems. *Megalopta* bee eyes
2653 are ~28 times more sensitive to light than the ones of *A. mellifera* (Greiner et al., 2004; Warrant
2654 et al., 2004). The photoreceptors of *Megalopta* bee possess an increased gain of transduction,
2655 but code less information when compared to diurnal species (Frederiksen et al., 2008). While
2656 this adaptation improves visual reliability, it compromises temporal resolution (Warrant and
2657 Dacke, 2011). Therefore, it is likely that crepuscular bees reliably detect the position of the
2658 light at the beginning of the trajectory and calculate the shortest path towards it. However,
2659 slower movements might be necessary to adjust temporal resolution and image reliability, as

2660 *M. aegis* gradually approaches the light source. A similar finding was shown for the flight of
2661 *M. genalis* (Baird et al., 2011). *Megalopta genalis* flies slower compared to bumblebees, this
2662 is probably because *M. genalis* use temporal summations to help them perceive optical flow
2663 and use it for flight control (Baird et al., 2011).

2664 Whereas wavelength clearly modulated phototaxis in crepuscular bees, light irradiance
2665 did not significantly influence the average duration or length of the phototactic paths in *M.*
2666 *aegis*. Contrarily, studies in honeybees using an equivalent range of green-light intensities
2667 found significant effects of irradiance on the duration and length of the phototactic paths (Erber
2668 and Sheiner, 2006; Scheiner et al., 2014). This difference could be related to the larger
2669 acceptance angles and receptive fields of the photoreceptors of crepuscular bees, which enhance
2670 photon capture and combined to other neural summation mechanisms, increase sensitivity to
2671 light (Greiner et al., 2004ab; Warrant et al., 2004; Frederiksen et al., 2008). This increased light
2672 sensitivity allows crepuscular bees to easily detect low light intensities. Maybe, the whole range
2673 of light intensity used in our experiments was above the threshold necessary to promote a
2674 comparable pattern of phototactic response in crepuscular bees. Further studies using a lower
2675 range of light intensity would be necessary to test this hypothesis.

2676 Although average duration and length of paths did not vary with light intensity, we
2677 found an interesting effect of light intensity on the speed dynamics of phototactic paths in *M.*
2678 *aegis*. When light irradiance increased, the mean instantaneous speed visibly increased to UV,
2679 slightly increased to green and conversely, decreased to blue light. Light intensity significantly
2680 modulated the speed of phototactic orientation in crepuscular bees, but in a different way for
2681 each specific wavelength. Curiously, we found that the phototactic paths of crepuscular bees to
2682 blue light not only became slower, but also became more tortuous (higher deviation angles) as
2683 irradiance increased. Since blue light excites not only M photoreceptors, but also the
2684 extremities of the absorption curves of the S and L photoreceptors (Peitsch et al., 1992), the
2685 equivalent metrics found in phototactic paths to blue and green lights could raise the suggestion
2686 that phototaxis to these lights is modulated by L or S+L excitation. However, the particularities
2687 here found in the phototaxis dynamics of *Megalopta* towards blue light reinforce the hypothesis
2688 that all three different photoreceptor channels, including the M receptor, contribute to
2689 phototaxis in crepuscular bees. Future experiments analyzing the phototaxis of crepuscular bees
2690 to diverse combinations of monochromatic lights may help to better understand how

2691 information of distinct photoreceptor channels interact in the brain of crepuscular bees during
2692 this behavior.

2693

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2818 CONCLUSÃO GERAL

2819 Ao longo do desenvolvimento desta tese foram estudados vários aspectos
2820 comportamentais e morfológicos das abelhas crepusculares. Foi mostrado quantitativamente,
2821 pela primeira vez, que é vantajoso para as abelhas crepusculares explorarem as flores
2822 quiropterófilas no curto período ao anoitecer e amanhecer. Devido à sua capacidade de voar em
2823 horários com baixa intensidade de luz, em alguns anoiteceres, as *Ptiloglossa* foram as primeiras
2824 a coletar o pólen das flores novas de *P. longiflorum*. Ao amanhecer, após a visita dos morcegos,
2825 as anteras de *P. longiflorum* ainda estavam ricas em pólen. Dessa forma, mais uma vez as
2826 *Ptiloglossa* as exploram por 20 minutos sem a presença de outro visitante floral. Como
2827 consequência dessas visitas sem competidores, as abelhas crepusculares removiam mais pólen
2828 por minuto do que as abelhas diurnas e os visitantes noturnos.

2829 Ao amanhecer, as abelhas *Ptiloglossa* usam a combinação dos odores florais e cores dos
2830 estames das flores de *P. longiflorum* como pista para as encontrar. Além disso, os odores florais
2831 são responsáveis por guiar essas abelhas até as flores. Embora as abelhas crepusculares tenham
2832 pousado em algumas flores apenas com a pista visual, não ficou claro qual pista desencadeia o
2833 comportamento de coleta de recursos nessas abelhas. Diferente das *Ptiloglossa*, as abelhas
2834 diurnas *Xylocopa grisescens* usam principalmente a cor dos estames de *P. longiflorum* para
2835 encontrar as flores. Para essas abelhas, tal pista visual é importante tanto para guiá-las até as
2836 flores, como também para desencadear o comportamento de coleta de recurso. É provável que
2837 a diferença no uso de pistas florais pelas abelhas *Ptiloglossa* e carpinteiras esteja relacionado
2838 com a intensidade luminosa durante o forrageamento e com os compostos florais majoritários
2839 de *P. longiflorum*. As *Ptiloglossa* buscam por essas flores 20 minutos antes das *X. grisescens*,
2840 período que 126 vezes mais escuro que o horário de início da visita das abelhas carpinteiras.
2841 Assim, sugerimos que a integração dos estímulos visuais e olfativos pelas *Ptiloglossa* torna as
2842 pistas florais mais confiáveis e facilita a busca por flores ricas em recursos durante o crepúsculo.
2843 Já as abelhas carpinteiras visitam as flores no início do amanhecer. À medida que a intensidade
2844 luminosa aumenta, aumenta também a confiabilidade do sinal visual, o que possibilita às
2845 abelhas encontrar flores usando apenas as cores dos estames como pista. Além disso, os odores
2846 produzidos pelas flores de *P. longiflorum* não são comuns em flores melitófilas visitadas por
2847 abelhas diurnas ao longo de todo o dia. Dessa forma, sugerimos que os odores florais de *P.*
2848 *longiflorum* são pouco atrativos para as abelhas carpinteiras.

2849 Para que as abelhas crepusculares consigam explorar as flores em horários pouco
2850 iluminados, elas possuem uma série de adaptações no sistema visual que as possibilita ver no
2851 escuro. Além do tamanho das estruturas que compõem o sistema visual dessas abelhas ser
2852 influenciado pelo hábito, essas estruturas também estão correlacionadas com o seu tamanho
2853 corporal. A exceção são as facetas frontais, que possuem o mesmo diâmetro em abelhas
2854 crepusculares de diferentes tamanhos corporais. Dessa forma, abelhas crepusculares pequenas
2855 possuem o diâmetro das facetas tão grandes quanto as abelhas crepusculares grandes. Essa
2856 região dos olhos possui um papel importante no processamento da paisagem, localização das
2857 flores e diversos outros tipos de interações ecológicas envolvendo a visão. Assim, é provável
2858 que ao longo da história evolutiva tenham sido selecionados indivíduos com menor tamanho
2859 corporal, mas com facetas frontais grandes, o que aumenta a sensibilidade à luz, permitindo que
2860 essas abelhas pequenas forragearem no crepúsculo.

2861 Diferentemente do que é descrito para as abelhas diurnas, as abelhas crepusculares nem
2862 sempre são atraídas aos estímulos luminosos. A redução na atração a luz pode ter sido uma
2863 mudança importante ao longo da transição entre o hábito diurno para o crepuscular/noturno.
2864 Quando há a resposta a luz, ela é mais forte a luz UV do que azul e verde. Esses resultados
2865 sugerem que o fotorreceptor S, que absorve luz na região do UV, tem um papel mais relevante
2866 na fototaxia que os fotorreceptores M e L, que absorvem luz principalmente na região do azul
2867 e verde.