Temporal changes in the most effective pollinator of a bromeliad pollinated by bees and hummingbirds

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A generalist pollination system may be characterized through the interaction of a plant species with two or more functional groups of pollinators. The spatiotemporal variation of the most effective pollinator is the factor most frequently advocated to explain the emergence and maintenance of generalist pollination systems. There are few studies merging variation in floral visitor assemblages and the efficacy of pollination by different functional groups. Thus, there are gaps in our knowledge about the variation in time of pollinator efficacy and frequency of generalist species. In this study, we evaluated the pollination efficacy of the floral visitors of *Edmundoa lindenii* (Bromeliaceae) and their frequency of visits across four reproductive events. We analysed the frequency of the three groups of floral visitors (large bees, small bees, and hummingbirds) through focal observations in the reproductive events of 2015, 2016, 2017, and 2018. We evaluated the pollination efficacy (fecundity after one visit) through selective exposure treatments and the breeding system by manual pollinations. We tested if the reproductive success after natural pollination varied between the reproductive events, and also calculated the pollen limitation index. *Edmundoa lindenii* is a self-incompatible and parthenocarpic species, requiring the action of pollinators for sexual reproduction. Hummingbirds had higher efficacy than large bees, and small bees acted only as pollen larcenists. The relative frequency of the groups of floral visitors varied between the reproductive events. Pollen limitation has occurred only in the reproductive event of 2017, when visits by hummingbirds were scarce and reproductive success after natural pollination was the lowest. We conclude that hummingbirds and large bees were the main and the secondary pollinators of *E. lindenii*, respectively, and that temporal variations in the pollinator assemblages had effects on its reproductive success. Despite their lower pollination efficacy, large bees ensured seed set when hummingbirds failed. Thus, we provide

evidence that variable pollination environments may favour generalization, even under differential effectiveness of pollinator groups if secondary pollinators provide reproductive assurance.

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2 **hummingbirds**

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19 **ABSTRACT**

20 **Background**

21 A generalist pollination system may be characterized through the interaction of a plant species

22 with two or more functional groups of pollinators. The spatiotemporal variation of the most

23 effective pollinator is the factor most frequently advocated to explain the emergence and

24 maintenance of generalist pollination systems. There are few studies merging variation in floral

25 visitor assemblages and the efficacy of pollination by different functional groups. Thus, there are

26 gaps in our knowledge about the variation in time of pollinator efficacy and frequency of

27 generalist species. In this study, we evaluated the pollination efficacy of the floral visitors of

28 *Edmundoa lindenii* (Bromeliaceae) and their frequency of visits across four reproductive events.

29 **Methods**

30 We analysed the frequency of the three groups of floral visitors (large bees, small bees, and

31 hummingbirds) through focal observations in the reproductive events of 2015, 2016, 2017, and

32 2018. We evaluated the pollination efficacy (fecundity after one visit) through selective exposure

33 treatments and the breeding system by manual pollinations. We tested if the reproductive success

34 after natural pollination varied between the reproductive events, and also calculated the pollen

35 limitation index.

36 **Results**

37 *Edmundoa lindenii* is a self-incompatible and parthenocarpic species, requiring the action of

38 pollinators for sexual reproduction. Hummingbirds had higher efficacy than large bees, and small

39 bees acted only as pollen larcenists. The relative frequency of the groups of floral visitors varied

40 between the reproductive events. Pollen limitation has occurred only in the reproductive event of

41 2017, when visits by hummingbirds were scarce and reproductive success after natural

42 pollination was the lowest.

43

44 **INTRODUCTION**

45 In most plants, flowers are visited by a diverse assemblage of animals, which characterizes

46 generalist pollination systems (*Waser et al., 1996*). Based on the behavior and

47 morphophysiological traits, floral visitors can be arranged in different functional groups of

48 pollinators, which may differ in their contribution to the plant reproductive success (*Fenster et*

49 *al., 2004*). This difference in contribution occurs as these pollinators can vary in frequency of

50 visits and ability to transfer pollen (*Shuttleworth & Johnson, 2008; Ollerton, 2017*). Studies

51 encompassing generalist pollination systems mainly report floral visitor assemblages and

52 visitation rates (*e.g., Thompson, 2001; Freitas & Sazima, 2006; Scrok & Varassin, 2011*);

53 despite not all visitors are actual pollinators (*Armbruster, Fenster & Dudash, 2000; Ollerton,*

54 *2017*). Therefore, studies that evaluate the pollination efficacy (for instance, measuring seed set

55 after one visit, *Freitas, 2013*) of different functional groups of pollinators are necessary to better

56 understand pollinators' relevance on plants with a generalist pollination system.

57   Based on the principle of "the most effective pollinator," a plant having more than one

58 functional group of pollinators could be interpreted as an intermediary stage in the shift from one

59 specialized pollinator to another (*Stebbins, 1970*). An alternative explanation is that generalist

60 systems are not transient and may be favored in certain scenarios, for example, under

61 unpredictable pollination environments (*Herrera, 1988; 1996; Waser et al., 1996; Armbruster et*

62 *al., 2000; Gómez & Zamora, 2006; Ollerton et al., 2007*). Accordingly, the spatiotemporal

63   variation of the most effective pollinator is the factor most frequently advocated to explain the

64   emergence and maintenance of generalist pollination systems. Several studies have explored

65   temporal variation in composition and frequency of floral visitors (*Fenster & Dudash, 2001;*

66   *Ivey, Martinez & Wyatt, 2003; Zych et al., 2018*). Other studies have quantified the effectiveness

67   (i.e., the product of efficacy times visitation rate, after *Herrera, 1987*; *Freitas 2013*) of different

68   pollinator species or functional groups (e.g., *Amorim, Galetto & Sazima, 2013; Salas-Arcos,*

69   *Lara & Ornelas, 2017*). However, information about floral visitor assemblages and their

70   effectiveness on pollination over multiple reproductive events is restricted to a few systems

71   (*Herrera, 1987; 1988; Larsson, 2005; Wiggam & Ferguson, 2005*). Thus, there are gaps in

72   knowledge about the variation of pollinators in generalist plants and their effectiveness over

73   time.

74   Generalized pollination systems have ecological and evolutionary dimensions

75   (*Armbruster et al., 2000*), therefore the effect of several pollinators in the process of evolutionary

76   generalization depends on the selective pressures exerted by those floral visitors. In this sense,

77   differences in pollination efficacy (sensu *Freitas, 2013*) among functional groups may be

78   enhanced if the variations in the pollination environment affect the plant reproductive success.

79   For instance, pollen limitation (PL), the lower fruit and/or seed production due to inadequate

80   pollen receipt, is widespread in angiosperms (*Ashman et al., 2004; Knight et al., 2005*), and

81   similarly to the pollinator effectiveness, its magnitude varies at several scales (*Bennett et al.,*

82   *2018*). However, how temporal variations in the pollination environment and PL levels are

83   related is a fundamental but poorly understood aspect to a better understanding of the

84   mechanisms that lead to the maintenance of generalized pollination systems (see *Koski et al.,*

85   *2018*).

86   Bromeliaceae is the largest family of predominantly Neotropical angiosperms (*Givnish,*

87   *2017*) and hummingbirds are the main group of pollinators of them (*Benzing, 2000*). However,

88   several species are known to have mixed pollination systems, for example involving

89   hummingbirds and bees or bats (*Givnish et al., 2014*). *Edmundoa lindenii* (Regel) Leme bears

90   flowers that are visited by both hummingbirds and bees in montane tropical forests, however we

91   do not know the role of those groups on the plant sexual reproduction over time. Here, we

92   evaluated the efficacy of three groups of floral visitors and their frequency of visits over four

93   reproductive events. In particular, we addressed the following questions: (1) Do hummingbirds,

94 large bees and small stingless bees pollinate this species, considering the high divergence of
95 traits between them? (2) Is the pollination efficacy of hummingbirds higher than large bees, since
96 hummingbirds pollinate most species of Bromeliaceae? (3) Are the relative and gross frequencies
97 of floral visits by each group equivalent over four reproductive events, since clear environmental
98 variations were not noted in those years? (4) However, if they are not equivalent, are those
99 variations in frequency related to the reproductive success in natural conditions and the
100 occurrence of pollen limitation?

101 **MATERIALS AND METHODS**

102 **Study site and species**

103 This study was conducted in an area covered by montane Atlantic Forest, located in the Serra dos
104 Órgãos National Park (PARNASO), Rio de Janeiro state, Brazil (22° 52'- 22° 54' S and 42° 09'-
105 45° 06' W, ca. 960 m a.s.l.) among four reproductive events (from 2014 to 2018). The average
106 annual rainfall at the study site is 2,436 mm, with the rainiest period between December and
107 March and colder and drier months from June to August. The mean annual temperature is 18.6
108 °C, with minimum and maximum monthly temperatures of 13.7 °C and 22.9 °C (climate data for
109 2015 to 2018 from the meteorological station located inside the PARNASO). The field research
110 reported here was performed using the required permit (SISBIO No. 34882, No. 432793).

111   *Edmundoa lindenii* (Regel) Leme (Bromeliaceae - Bromelioideae) is a terrestrial,
112 saxicolous, or epiphyte herb, endemic to the Atlantic Forest in south and southeastern Brazil
113 (*Martinelli et al., 2008; BFG, 2015*). In the study area, this species blooms between December
114 and February and produces fruits between March and April; its flowers are visited by bees and
115 hummingbirds (*R.L.B. Leal et al., pers. obs.*).

116

117 **Floral biology**

118 We measured inflorescences of *E. lindenii* (n = 16 individuals) directly in the field with a digital
119 calliper, considering the following traits: scape length, inflorescence diameter, and bract length.
120 Flowers (n = 73) from 28 individuals were collected in the field, stored in 70% alcohol, and
121 measured in the laboratory with a digital calliper considering the following structures: corolla
122 tube length (i.e., from septal nectary to the opening of the corolla) and the width of the corolla
123 tube opening. We counted the number of ovules in 25 flowers (n = 15 individuals).

124   To analyse the colour quantitatively, we measured the spectral reflectance of petals,

125 sepals, and bracts. For this, 12 flowers (n = 6 individuals) were collected in the field, stored in

126 thermal bags containing moist paper, and brought to the laboratory, where they were

127 immediately measured (*Lunau et al., 2011*). We measured the reflectance using an USB2000

128 spectrophotometer (OceanOptics, Inc., Dunedin, FL, USA) coupled with a deuterium–halogen

129 light source (DH-2000; OceanOptics, Inc., Ostfildern, Germany), with a light emission range

130 between 215 and 1700 nm. We took all reflectance measurements at a 45° angle in relation to the

131 plant structure, and we used barium sulphate as the white standard and black paper as the black

132 standard (*Chittka & Kevan, 2005*).

133   We used the logarithm version of the receptor noise-limited model (*Vorobyev et al.,*

134 *2001*) to compare the colours of the petals, sepals, and bracts. Chromatic distances were

135 calculated according to the trichromatic formulation for bees and the tetrachromatic formulation

136 for hummingbirds (*Vorobyev & Osorio, 1998*). We modelled spectral sensitivity curves using

137 data from *Sephanoides sephaniodes* (*Herrera et al., 2008*) to estimate hummingbird colour

138 distances, and from *Bombus terrestris* for bees *(Telles & Rodriguéz-Gironés, 2015*). In all cases,

139 we used standard daylight illumination (D65 – *Wyszecki & Stiles, 1982*). Using these models, we

140 determined the spectral location of each structure in a colour space for each pollinator.

141   The distance between two points in a colour space provides an approximation of the

142 perceived colour difference (*Endler & Mielke, 2005*). We evaluated colour distances between

143 sepals, petals, and bracts. Using the receptor noise-limited model, we estimated that two colours

144 were discriminable if their distance was greater than 0.27 units for bees (*Telles & Rodríguez-*

145 *Gironés, 2015*) and 1.0 for hummingbirds (*Vorobyev et al., 1998*). For representation, we also

146 calculated the colour loci of the flower colours in the respective colour space models: the colour

147 hexagon for bees (*Chittka* 1992) and the colour tetrahedron for hummingbirds (*Vorobyev et al.,*

148 *1998*).

149

150 **Nectar**

151 We measured the nectar volume in flowers previously bagged in bud stage, with a graduated

152 microliter syringe (Hamilton, Nevada, USA), and the concentration with hand-held refractometer

153 (Bellingham + Stanley Eclipse, UK). To evaluate nectar production during anthesis, without the

154 effect of nectar removal, 36 flowers (n = 10 individuals) previously bagged at the bud stage were

155   measured once after anthesis onset. In total, we performed measurements at four different times

156   of the day: 7:00 am (n = 10 flowers); 8:30 am (n = 10); 10:00 am (n = 10); and 11:30 am (n = 6).

157   To evaluate if nectar removal stimulates its secretion, 24 flowers were submitted to four

158   treatments (n = 6 individuals, four flowers per individual – one flower per treatment per

159   individual): R = nectar was measured once at 11:00 am; R1 = nectar was measured twice (at

160   10:00 am and 11:30 am); R2 = nectar was measured three times (at 8:30 am, 10:00 am and 11:30

161   am); and R3 nectar was measured four times (at 7:00 am, 8:30 am, 10:00 am and 11:30 am).

162   Thus, for the treatments R1, R2, and R3, nectar was remeasured on the same flowers. We

163   calculated the total amount of sugar (mg) per flower by multiplying nectar volume (μL) by its

164   corrected concentration (mg/μL) according to *Dafni, Kevan & Husband* (*2005*).

165   **Breeding system and pollen limitation**

166   We evaluated the breeding system and pollen limitation (PL) through manual pollination

167   treatments. Floral buds of different individuals were previously bagged with "voile" bags, and

168   the flowers submitted to the following treatments: (1) spontaneous self-pollination - 20 flowers

169   from 8 individuals, were bagged and not manipulated in 2016; (2) hand self-pollination - 49

170   flowers from 20 individuals, were supplemented manually with pollen from the same flower and

171   bagged in 2016; (3) hand cross-pollination - 130 flowers from 47 individuals, were supplemented

172   with pollen from other individual, located at least 10 m away, and then bagged. We conducted

173   the cross-pollination treatment in the years 2016 (26 flowers), 2017 (48 flowers), and 2018 (56

174   flowers). (4) Pollination under natural conditions - 131 flowers from 36 individuals were marked

175   and kept unbagged. We evaluated the flowers from natural pollination in 2016 (49 flowers), 2017

176   (20 flowers), and 2018 (62 flowers). At the end of the experiments we collected the fruits and

177   counted the number of seeds per fruit in the laboratory. We calculated the index of pollen

178   limitation as IPL = 1 - P_n/P_c where P_n is the proportion of seed-bearing fruits multiplied by the

179   mean number of seeds per fruit of flowers exposed to natural pollination, and P_c is the

180   proportion of seed-bearing fruits multiplied by the mean number of seeds per fruit of flowers

181   after hand cross-pollination (adapted from *Lloyd and Schoen,* 1992; *Larson and Barrett, 2000*).

182   We used as response variable of reproductive success in all analyses such an estimate combining

183   fruits bearing seeds and the number of seeds, which is appropriate because *E. lindenii* is

184   parthenocarpic (i.e., flowers develop into fruits independent of pollination). Values of IPL ≤ 0.2

185   indicate absence of PL, whereas IPL > 0.8 indicates strong PL (*Freitas, Wolowski & Sigiliano,*

186   *2010*). We assessed the self-incompatibility by the index of incompatibility (ISI), a relative

187   measure of the seeds produced after self- and cross-pollination (*Zapata and Arroyo, 1978*).

188   Species with ISI < 0.30 may be classified as self-incompatible (*Ramirez & Brito, 1990*).

189

190   **Frequency and efficacy of floral visitors**

191   We performed focal observations (sensu *Dafni et al., 2005*) to evaluate the identity of floral

192   visitors and their frequency of visits, by censuses of 30-min per individual (n = 190 individuals)

193   between 6:00 am and 12:00 am, totalizing 184 hours of observation. Observations were done in

194   four reproductive events, in the years 2015 (43.5 hours, n = 50 individuals), 2016 (39.0 hours, n

195   = 42), 2017 (51.0 hours, n = 48), and 2018 (50.5 hours, n= 50). Images and videos were captured

196   during the visits to evaluate the foraging behaviour and the floral resources obtained. The visits

197   were identified as legitimate or illegitimate by the expected mode of pollination, considering the

198   shape and arrangement of the flower parts (sensu *Irwin et al., 2010; Freitas, 2018*). Specimens

199   of insects were collected for posterior identification. We grouped the floral visitors into three

200   functional groups based on identity, body size and foraging behaviour, as following:

201   hummingbirds, large bees (length ≥ 10 mm), and small bees (< 10 mm).

202   We evaluated the efficacy of the three functional groups of floral visitors through

203   experiments of selective exposition. In this experiment, flowers previously bagged at the bud

204   stage were exposed to a single visit by any visitor of the three functional groups, as follows:

205   small bees (48 flowers in 2016), large bees (20 flowers in 2017 and 60 in 2018), and

206   hummingbirds (20 flowers in 2017 and 65 in 2018). Here we also considered the proportion of

207   seed-bearing fruits multiplied by the mean number of seeds per fruit as the response variable of

208   the pollination efficacy by each functional group.

209

210   **Data analyses**

211   We performed all the analyses in R version 3.4.4 (*R Development Core Team, 2018*). We

212   evaluated the production of nectar during anthesis and the effect of nectar removal in nectar

213   secretion by analyses of variance (one-way ANOVA), using the function *aov*. We assessed the

214   differences between treatments (time of anthesis and number of removals) by Tukey HSD post-

215   hoc test, using the function *TukeyHSD*.

216   We conducted a linear model to evaluate if the reproductive success after natural

217   pollination varied between three reproductive events (2016, 2017, and 2018). Prior to analyses,

218   we cubic-root transformed values of the response variable to meet the normality assumptions.

219   We used the reproductive events (three levels: 2016, 2017, and 2018) as fixed effect. We

220   established the model using the function *lm* and we tested the model assumptions by visual

221   inspection of the residuals using the *qqnorm* function. We calculated the significance of each

222   term in the model using the function *Anova* (Type II) from the *car* package *(Kuznetsova et al.,*

223   *2017)* and the differences between levels of categorical factors using the function *lsmeans* from

224   *lsmeans* package (*Lenth, 2016)*.

225   To evaluate whether hummingbirds and large bees differ in their efficacy, we conducted a

226   linear model. Prior to analyses, we cubic-root transformed values of the response variable to

227   meet the normality assumptions. We used the functional group of pollinators (two levels:

228   hummingbirds and large bees) and the year when the treatments were conducted (two levels:

229   2017 and 2018) as fixed effects. We established the model using the function *lm* and we tested

230   the model assumptions by visual inspection of the residuals using the *qqnorm* function. We

231   calculated the significance of each term in the model using the function *Anova* (Type II) from the

232   *car* package (*Kuznetsova et al., 2017*) and the differences between levels of categorical factors

233   using the *lsmeans* package (*Lenth, 2016)*. We did not compare the efficacy of small bees as no

234   seeds were produced after their visits.

235   To evaluate if the frequency of visits varies between the four reproductive events and the

236   group of floral visitors, we conducted a log-linear model for categorical data (*Wood*, 2017). We

237   used the relative frequency of visits as a response variable, and the reproductive events (four

238   levels: 2015, 2016, 2017, and 2018) and the functional groups (three levels: hummingbirds, large

239   bees, and small bees) as fixed effects. We established the model using the *glm* function with the

240   family set to *poisson* (*Lindsey, 1997*). We tested the model assumptions by visual inspection of

241   the residuals. We calculated the significance of each term in the model using the *anova* function

242   (test = *Chisq*) and the differences between levels of categorical factors using the *emmeans*

243   function available in the *emmeans* package.

244

245   **RESULTS**

246   **Floral biology**

247 The flowers of *E. lindenii* are grouped in a compound corymboid inflorescence with *ca.* 100–150

248 flowers, inserted in the leaf rosette (*Fig. 1*). Inflorescence diameter reached 121.32 ± 17.01 mm

249 and scape length 296.87 ± 23.86 mm (mean ± SD throughout the text). The flowers are

250 hermaphrodite, with the androecium presenting six stamens included in the corolla and anthers

251 with longitudinal dehiscence (*Fig. 1*). The gynoecium is also included in the corolla and the style

252 ends in a three-lobed stigma (*Fig. 1*). The inferior and trilocular ovary contained 197.9 ± 54.12

253 ovules. The length of bracts and sepals was 55.15 ± 6.99 mm and 26.0 ± 4.0 mm, respectively.

254 The corolla is tubular (length: 17.95 ± 2.92 mm) with a narrow opening (3.11 ± 1.17 mm). The

255 flowers have diurnal anthesis, characterized by the presence of exposed pollen grains and

256 receptive stigma. The anthesis began at around 06:00 am and lasted for about six hours when

257 corolla closed. The secretion of nectar started in the beginning of anthesis, and it did not increase

258 over time (F = 0.44; df = 3; p = 0.726; *Fig. 2A*). However, the removal of nectar stimulated new

259 secretion (F = 6.632, df = 3, p = 0.00273, *Fig. 2B*).

260   The bracts reflect red wavelengths, whereas the corolla is UV-reflecting white, and the

261 sepals are UV-absorbing white (*Fig. 3*). The colour of petals, sepals, and bracts, as well as open

262 or closed flowers, is distinguishable by bees and hummingbirds. Flower colour was 1–7 times

263 above the discrimination criteria (0.27) for bee vision (petals-sepals 4 ± 1, bracts-sepals 4 ± 2)

264 and 5–15 times above the discrimination criteria (1.0) for hummingbirds (petals-sepals 8 ± 2,

265 bracts-sepals 12 ± 4, bracts-petals 15 ± 7).

266

267 **Breeding system and pollen limitation**

268 *Edmundoa lindenii* is self-incompatible (ISI = 0.08; *Table 1*) and parthenocarpic (*Table 1*),

269 requiring the action of pollinators for sexual reproduction. We observed that the reproductive

270 success after natural pollination varied between reproductive events (ANOVA: F=12.9, df = 2 p

271 < 0.001). The lowest reproductive success occurred in the reproductive event of 2017 (contrasts:

272 **2016-2017**: t = 5.050; df = 103; p <0.001; **2016-2018**: t = 2.100; df = 103; p = 0.095; **2017-2018**:

273 t = -3.840; df = 103; p < 0.001; *Fig. 4*). Pollen limitation was expressive only in 2017 (PL index:

274 2016 = -0.21, 2017 = 0.70, 2018 = -0.003).

275

276 **Floral visitors and temporal variation**

277   The flowers of *E. lindenii* were visited by 11 species of animals belonging to three functional

278   groups (hummingbirds, large bees, and small bees; *Table 2*). Hummingbirds were the group with

279   the highest species richness with seven species (*Table 2*; *Fig.1*). The small bee *T. spinipes* was

280   the only visitor that conducted illegitimate visits, resulting in damage of corolla and/or anthers

281   by chewing. Hummingbirds and large bees foraged for nectar acting as legitimate visitors, and

282   small bees collected pollen.

283   The pollinator functional group affected the reproductive success of *E. lindenii* (*Table 3*)

284   and hummingbirds had higher efficacy than large bees (contrast: t =3.015 df = 148, p = 0.003;

285   *Table 4; Fig. 5*). Small bees did not act as pollinators, as none of the flowers they visited

286   produced seeds. The frequency of visits varied between functional groups of floral visitors with

287   significant interaction between functional group and reproductive event (*Table 4*; *Fig. 6*).

288

289   **DISCUSSION**

290   The frequency of floral visitors' groups (hummingbirds, large bees, and small bees) varied

291   between reproductive events of *E. lindenii*, and this variation resulted in a reduction in the

292   natural pollination in one year, when pollen limitation was also recorded. The existence of year-

293   to-year changes in the composition of floral visitors has been found in several systems (*Schemske*

294   *& Horvitz, 1984; Traveset & Sáez, 1997; Price et al., 2005; Olesen et al., 2008; Petanidou et al.,*

295   *2008*), while in others, pollination efficacy between different years was studied (*Fishbein &*

296   *Venable, 1996; Stoepler et al., 2012*). However, there are fewer studies that consider both plant

297   reproductive success and variations in the pollinator assemblages along time (*Herrera, 1990;*

298   *Fleming et al., 2001; Salas-Arcos et al., 2017*). Thus, through observational and experimental

299   approaches, we have shown that the reproductive success of a generalist species responded to

300   temporal variation of its assemblage of pollinators.

301   Overall hummingbirds had higher efficacy and visitation frequency than large bees, so

302   they could be pointed as the main pollinators of *E. lindenii*. Hummingbirds also act as the main

303   pollinators in other species of Bromeliaceae (*Schmid et al., 2011*; *Magalhães et al., 2018*).

304   However, large bees did not always act as secondary pollinators of *E. lindenii*: they were the

305   main pollinators when the frequency of visits by hummingbirds was low. Pollinators belonging

306   to different functional groups may vary in their pollination effectiveness, exerting pressures

307   toward specialization (*Stebbins, 1970; Rosas-Guerrero et al. 2014*). Thus, evolution of a

308   generalized pollination system is expected when different pollinators play the same role as

309   selective agents or if the less effective pollinators provide reproductive assurance, offsetting

310   fluctuations of the most effective pollinators. This could be the case for large bees and *E.*

311   *lindenii*, as they can ensure sexual reproduction when hummingbirds fail. In short, our results are

312   consistent with the hypothesis that the maintenance of generalist pollination is related to the

313   existence of variable pollination environments (*Waser et al., 1996*) and support that combined

314   measurements of reproductive success and pollinator assemblages along time and space as an

315   interesting approach in this regard (see *Gómez & Zamora, 2006* for additional suggestions).

316   Curiously, there was no evidence of declining populations of hummingbirds in the study area in

317   2017, the causes of the drastic reduction of visits to the flowers of *E. lindenii* by hummingbirds

318   that year are completely unknown.

319   Despite the differences on effectiveness, flowers of *E. lindenii* are attractive to both

320   hummingbirds and bees. Floral visitors identify and select flowers using a variety of

321   characteristics, including size and color contrasts (*Papiorek et al., 2016*), and hummingbirds and

322   bees can detect different color spectra (*Chittka & Waser, 1997; Vorobyev & Osorio, 1998*). The

323   floral traits of *E. lindenii* are detectable by both groups of floral visitors. Petals had UV

324   reflection, sepals absorbed UV, and bracts were red. These results correspond to the expected

325   pattern for attraction of bees and hummingbirds, as bees have a spectrum of vision that includes

326   UV wavelengths, around 300–400 nm (*Kevan, Chittka & Dyer, 2001*), and hummingbirds are

327   known for their preference for red-coloured flowers that mostly are UV-absorbent (*Lunau et al.*

328   *2011*). However, trade-offs between selective pressures exerted by different pollinators may

329   occur if they differ in preference for floral traits (*Gervasi & Schiestl, 2017*). As pointed out

330   above, we may speculate that the temporal fluctuation in the visitor frequencies of *E. lindenii*

331   may reduce the probability of pollinators exerting consistent selective pressures on its floral traits

332   (*Schemske & Horvitz, 1984; Gómez & Zamora, 2006*). In this case, attractive and non-restrictive

333   flowers for large bees are important for reproductive assurance in reproductive events with a low

334   frequency of hummingbirds, thus it would not be expected a shift to specialized hummingbird-

335   flowers. However, further experiments are needed to support that selection toward pollination

336   specialization by hummingbirds is restrained in this system.

337   In addition to visual signals, the nectar of *E. lindenii* is accessible to hummingbirds and

338   large bees, thus its flowers are attractive and legitimately accessible to the two groups. The

339 dynamics of nectar production influences the behaviour of pollinators during visits to the flowers

340 (*Parachnowitsch, Manson & Sletvold, 2018*). Although nectar production of *E. lindenii* did not

341 increase over time, its removal stimulated further secretion. Similar results have been found in

342 other bromeliads, including some species visited by hummingbirds and bees (*Galetto &*

343 *Bernardello, 1992; Ordano & Ornelas, 2004*). Nectar secretion after withdrawal may favor

344 repeated visits to the flower. This is consistent with our results of the pollination efficacy

345 experiment in *E. lindenii.* Specifically, seed set after one visit by a large bee or hummingbird

346 was lower than seed set of flowers exposed to pollinators during the whole anthesis, indicating

347 that more than one visit is necessary to achieve maximum fecundity in this species. At last,

348 nectar secretion after removals and repeated visits by pollinators could be related to the male

349 component of the reproductive success (see *Ordano & Ornelas, 2004*).

350 The population of *E. lindenii* at PARNASO was self-incompatible, but self-compatibility

351 has been registered in other populations of this species (*Matallana et al., 2010*). Variations

352 between self- incompatibility and self-compatibility within species are common in plant

353 evolution and may indicate transitions between reproductive systems (*Igic, Lande & Kohn,*

354 *2008*). Studies have shown that compatibility barriers can be broken by genetic changes (such as

355 mutations) (e.g., *Sassa et al., 1997*), physiological factors, elevated temperatures, and stress (e.g.,

356 *Tezuka et al., 1997*), allowing for self-pollination. Moreover, breeding systems may be related to

357 the degree of pollination generalization, linking shifts in pollination and incompatibility systems.

358 For instance, Wessinger and Kelly (*2018*) found a relationship between self-compatibility and

359 attributes related to the attraction of hummingbirds, including red flowers and loss of floral

360 aroma and UV- absorbing pigments. In contrast, we found self-incompatibility and UV-

361 absorbing sepals in *E. lindenii* despite higher frequency of visits by hummingbirds. Self-

362 incompatibility in this species seems to work as a barrier to autogamous pollination, since small

363 bees access the anthers, make long visits to the flower, and manipulate the pollen. In fact,

364 pollinators usually do not operate independently of herbivores (florivores in this case), which

365 may generate a trade-off between the fitness functions by each kind of organism (*Ashman, 2002;*

366 *Gómez & Zamorra, 2006; Gélvez-Zúñiga et al., 2018*).

367

368 **CONCLUSION**

369   Our results allow us to conclude that hummingbirds and large bees were the main and the

370   secondary pollinators of *E. lindenii*, respectively. Moreover, small bees had a negative effect on

371   its reproduction. These results could indicate a higher degree of specialization of this system than

372   the apparent generalization considering floral visitor composition (see *Padyšáková et al., 2013*),

373   which would be in accordance with the most effective pollinator principle (*Stebbins, 1970*).

374   However, temporal variations in the pollinator assemblages had effects on reproductive success

375   of *E. lindenii*, leading to the occurrence of pollen limitation when visits by hummingbirds were

376   scarce. Despite their lower pollination efficacy, large bees ensured seed set when hummingbirds

377   failed. Plant pollination generalization has been associated with similar effectiveness by the

378   different pollinators (*Waser et al., 1996*). However, we provide evidence that variable pollination

379   environments may favour generalization, even under differential effectiveness of pollinator

380   groups if secondary pollinators provide reproductive assurance. This reinforces the idea of

381   different mechanisms driving the evolution of generalized pollination systems (*Schiestl, Balmer*

382   *& Gervasi, 2018*).

383

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389

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Figure 1

fig1 - *Edmundoa lindenii* flowers were visited by three functional groups: hummingbirds, large bees, and small bees.

(A) Inflorescence and flowers; (B) Visit by the hummingbird *Amazilia fimbriata*; (C) visit by the large bees *Bombus morio*; (D) visit by the small bee *Trigona spinipes*. All observations were made in the montane Atlantic Forest at Serra do Órgãos National Park, southeastern Brazil.

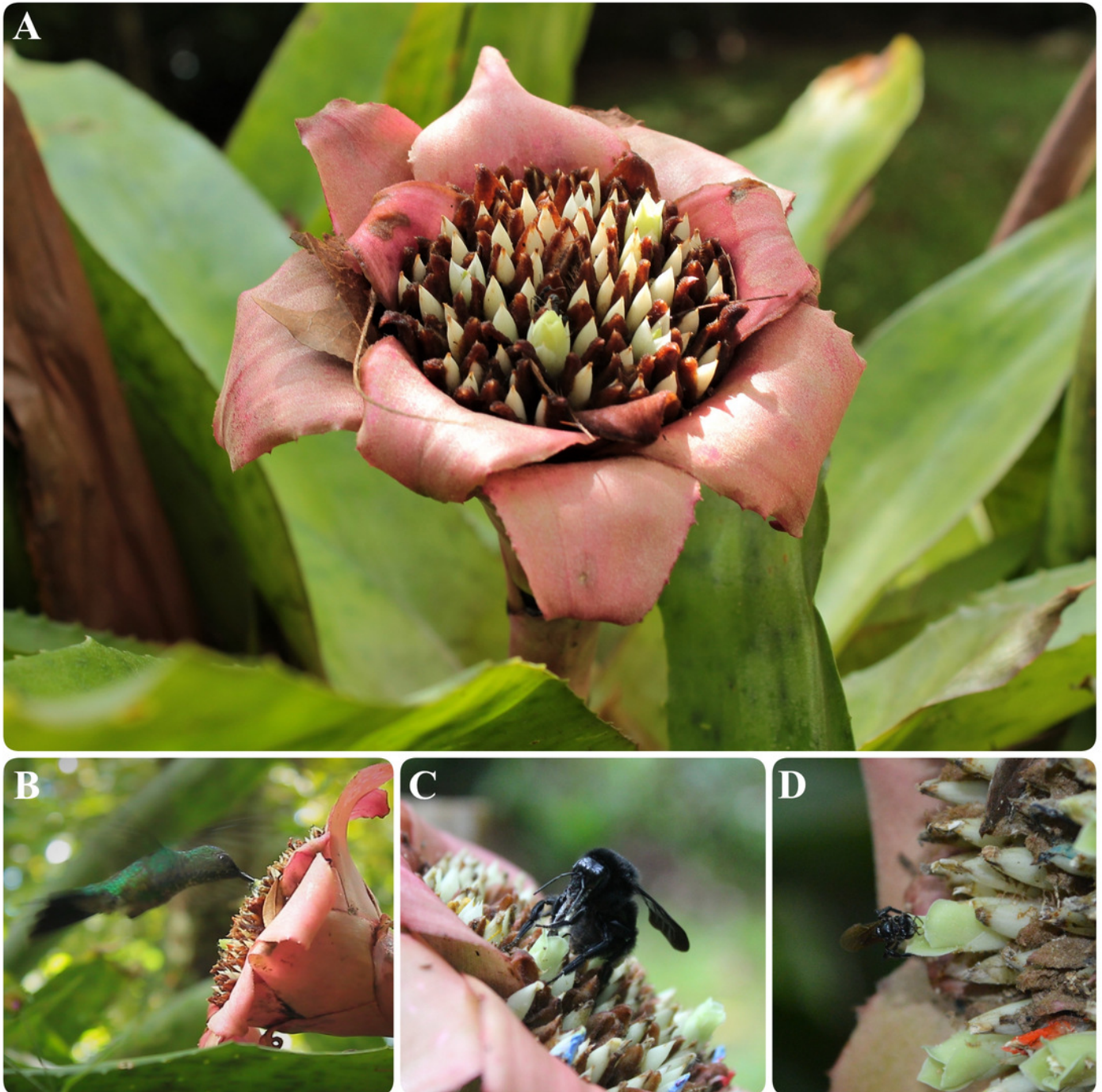



Figure 2

Nectar production in *Edmundoa lindenii* flowers did not increase during the anthesis, but the nectar removal stimulated new secretion.

(A) Nectar production along the anthesis at four different times of the day: 7:00h; 8:30h; 10:00h; and 11:30h. (B) Nectar production after experimental removal of nectar: R = no removal, sampled one time at 11:00h; R1 = one removal, sampled two times at 10:00h and 11:30h; R2 = two removals, sampled three times at 8:30h, 10:00h and 11:30h; and R3 = three removals, sampled four times at 7:00h, 8:30h, 10:00h and 11:30h. The horizontal line in the both boxplots represents the median values, the upper and lower sides of the box represent the corresponding quartiles, and vertical lines are minimum and maximum values of the data range. Dots are outliers. Different letters indicate statistical significance between pairs of years ($p < 0.05$) by ANOVA post-hoc test (TukeyHSD).

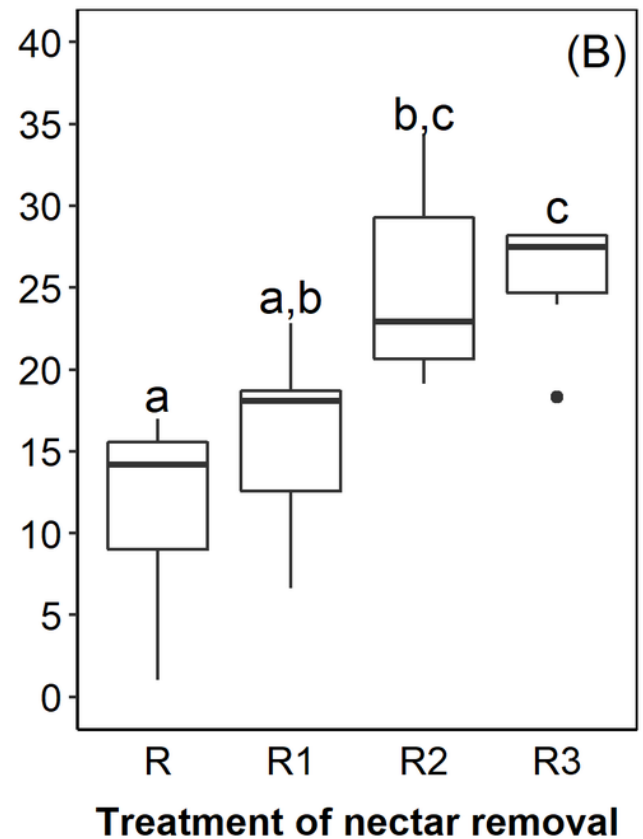
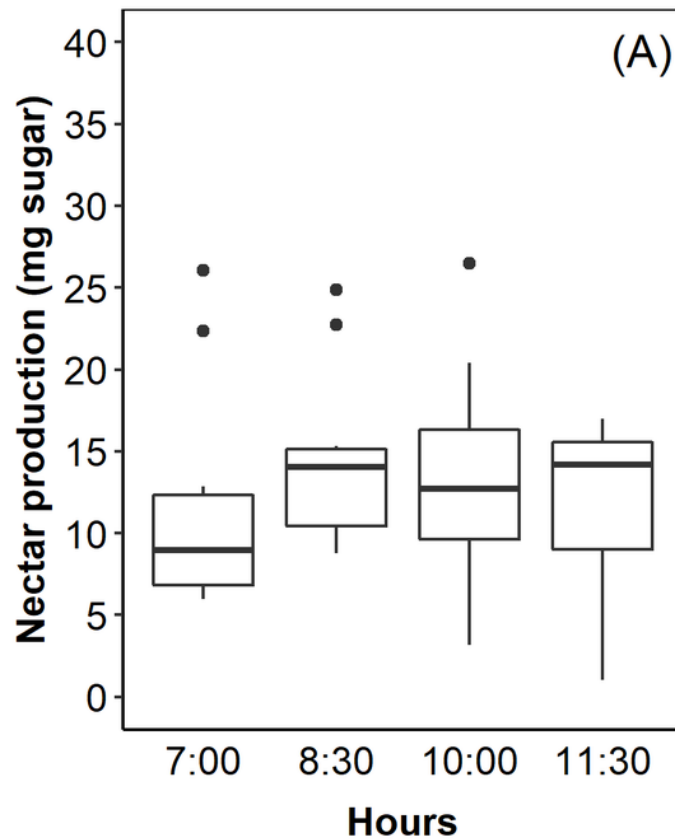



Figure 3

fig3 - Attractive structures of *Edmundoa lindenii* include red bracts, UV- reflecting white petals, and UV-absorbing white sepals, and can be detected by bees and hummingbirds.

(A) The reflectance spectra of attractive structures in *E. lindenii* inflorescences. For each structure, the colored line represents the mean reflectance and the corresponding color shading represents the standard deviation. Red = bract reflectance; blue = sepal reflectance; green = petal reflectance. (B) Hexagon model for bee vision based on the photoreceptors of *Bombus terrestris*. (C) Tetrahedron model for bird vision based in the photoreceptors of *Sephanoides sephaniodes*. In both models, the gray point represents achromatic center, the red point represents mean loci for bracts, the blue point indicates the mean loci for sepals, and the green point represents mean loci for petals.

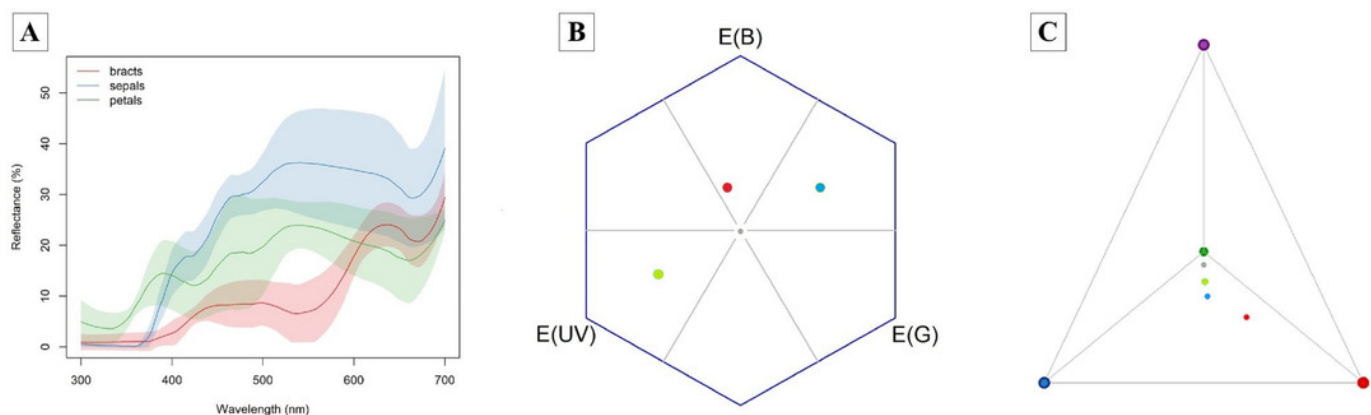



Figure 4

fig4 - The reproductive event of 2017 presented the lower reproductive success by natural pollination.

Reproductive success after natural pollination among three reproductive events (2016, 2017, and 2018). Seeds = proportion of fruits with seeds formed multiplied by the number of seeds in each fruit. Different letters show statistically significant difference ($p < 0.05$).

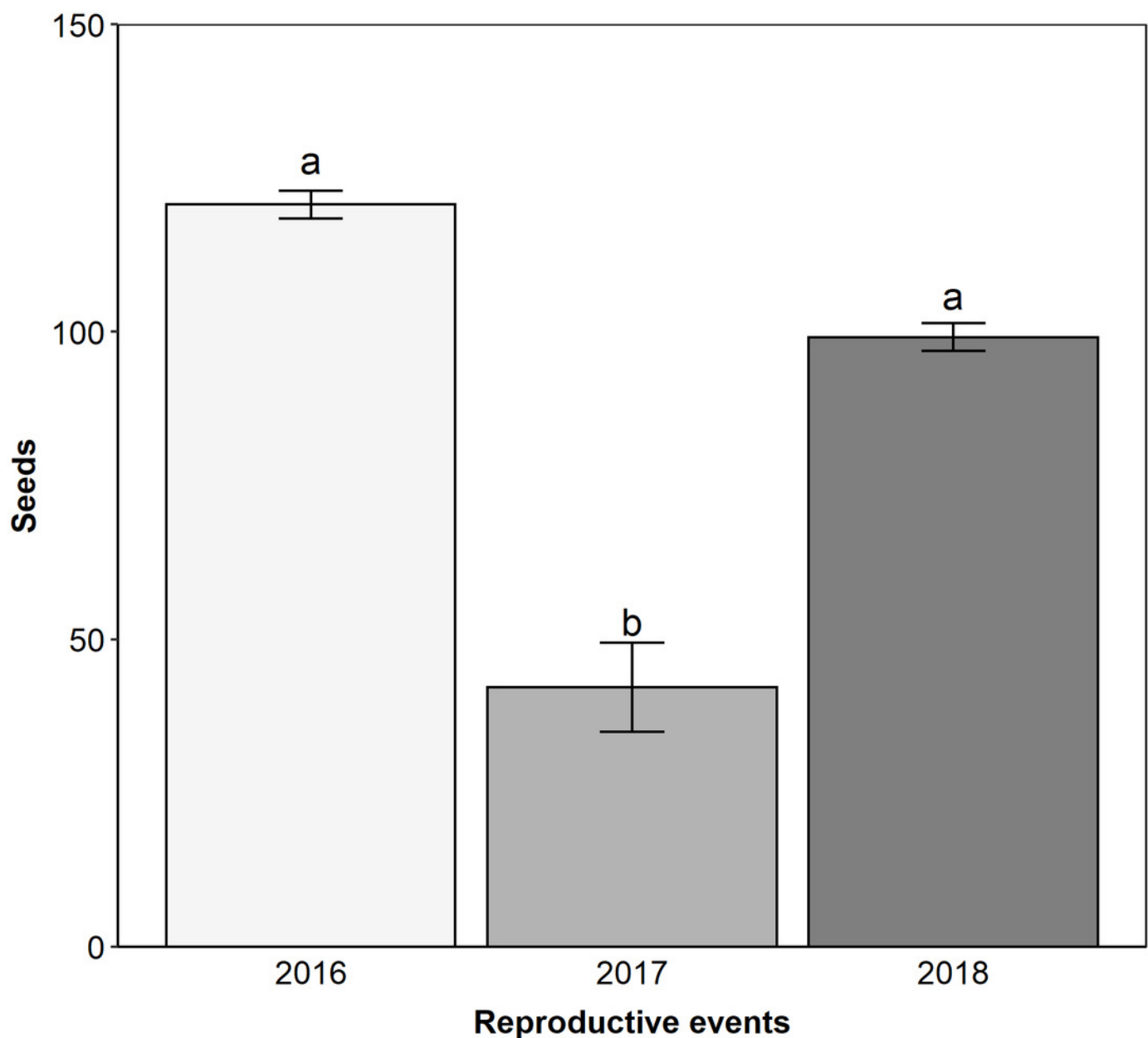



Figure 5

fig5A - Hummingbirds had higher efficacy than large bees in two reproductive events.

Pollinator efficacy in the reproductive events of 2017 (A) and 2018 (B). Seeds = proportion of fruits with seeds formed multiplied by the number of seeds in each fruit. Different letters show statistically significant difference ($p < 0.05$).

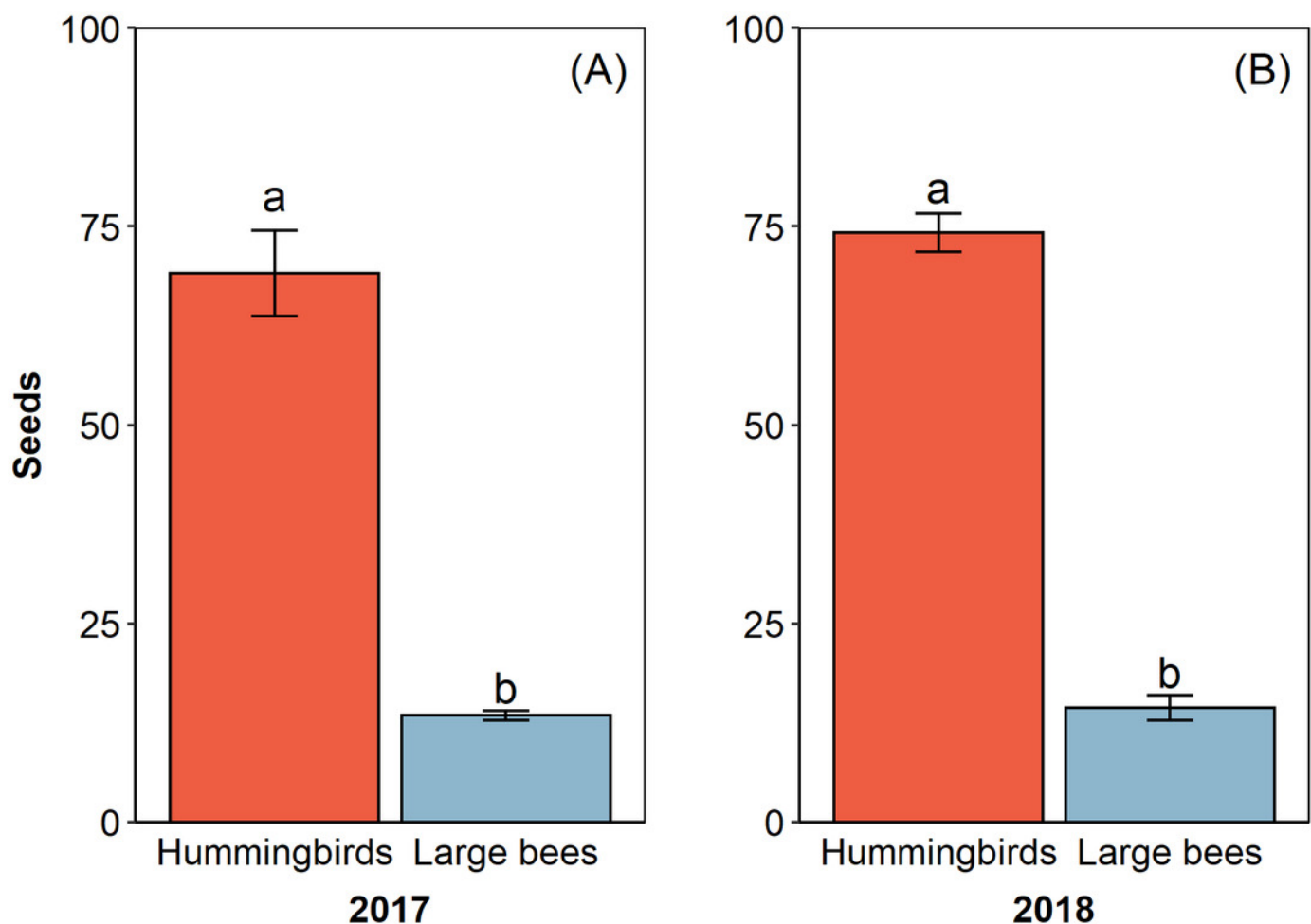
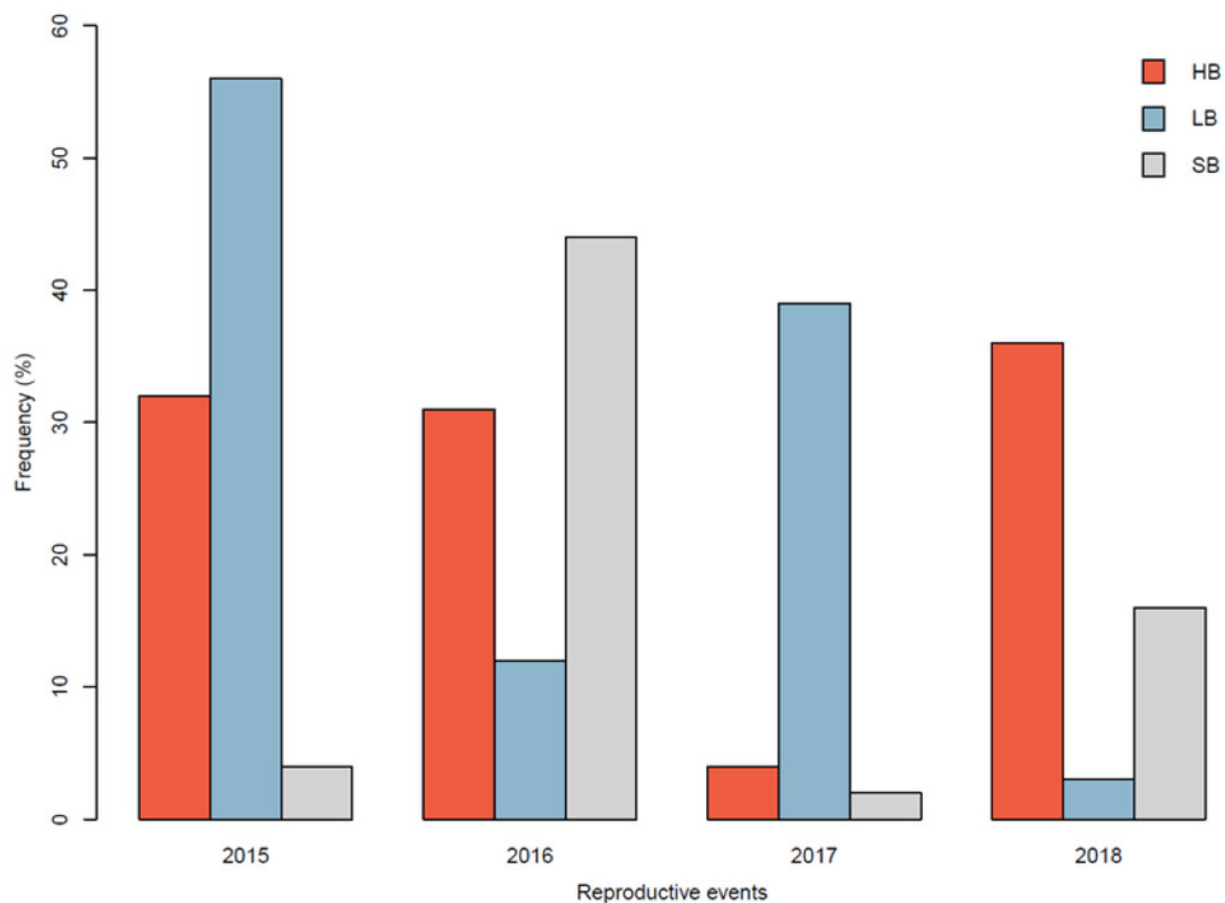



Figure 6

fig6 - Relative frequency of visits by each functional group in *Edmundoa lindenii* flowers varied among the four reproductive events.

The relative frequency of visits in the reproductive events of 2015, 2016, 2017 and 2018. HB = hummingbirds; LB = large bees; SB = small bees.

The population of *Edmundoa lindenii* in PARNASO is self-incompatible and parthenocarpic.

Fruit and seeds production after hand pollination treatments at Serra do Órgãos National Park, southeastern Brazil. Reproductive success = proportion of fruits with seeds multiplied by the number of seeds.

1 **Table 1:**

2 **The population of *Edmundoa lindenii* in PARNASO is self-incompatible and**

3 **parthenocarpic.**

4 Fruit and seeds production after hand pollination treatments at Serra do Órgãos National Park,

5 southeastern Brazil. Reproductive success = proportion of fruits with seeds multiplied by the

6 number of seeds.

Treatments	Flowers (n)	Fruits with seeds (n)	Fruit set	Seeds (mean ± sd)	Reproductive success
Spontaneous self-pollination	20	1	0.05	26	1.30
Manual self-pollination	49	4	0.08	126.00 ± 58.17	10.08 ± 4.65
Cross-pollination total	130	127	0.98	116.11 ± 58.97	113.64 ± 61.27
Cross-pollination 2016	26	23	0.89	126.57 ± 53.66	99.65 ± 57.91
Cross-pollination 2017	48	48	1.00	138.44 ± 63.47	138.44 ± 63.47
Cross-pollination 2018	56	56	1.00	98.88 ± 54.65	98.88 ± 54.65
Natural conditions total	131	108	0.82	119.08 ± 66.31	101.98 ± 58.43
Natural conditions 2016	49	45	0.92	131.19 ± 55.58	120.69 ± 51.13
Natural conditions 2017	20	11	0.55	76.73 ± 73.66	42.20 ± 40.51
Natural conditions 2018	62	52	0.84	118.04 ± 70.23	99.15 ± 58.99

Floral visitors and the resources they have taken in *Edmundoa lindenii* flowers.

All records were made in 2015 to 2018 at Serra do Órgãos National Park, southeastern Brazil. Functional groups: HB = hummingbirds, LB = large bees, SB = small bees. Type of resources: P = pollen, N = nectar.

1 **Table 2:**

2 **Floral visitors and the resources they have taken in *Edmundoa lindenii* flowers.**

3 All records were made in 2015 to 2018 at Serra do Órgãos National Park, southeastern Brazil.

4 Functional groups: HB = hummingbirds, LB = large bees, SB = small bees. Type of resources: P

5 = pollen, N = nectar.

Family	Species	Functional group	Resource
Trochilidae	*Amazilia lactea* (Lesson, 1832)	HB	N
	Amazilia versicolor (Vieillot, 1818)	HB	N
	Leucocholoris albicollis (Viellot, 1818)	HB	N
	Phaethornis eurynome (Lesson, 1832)	HB	N
	Ramphodon naevius (Dumont, 1818)	HB	N
	Thalurania glaucopis (Gmelin, 1788)	HB	N
	Amazilia fimbriata (Gmelin, 1788)	HB	N
Apidae	*Bombus morio* (Swederus, 1787)	LB	N/P
	Bombus brasiliensis (Lepeletier, 1835)	LB	N/P
	Euglossa sp.	LB	N
	Trigona spinipes (Fabricius,1793)	SB	P

The pollination efficacy of hummingbirds was higher than efficacy of large bees in *Edmundoa lindenii.*

ANOVA results on the pollination efficacy of functional groups of pollinators of *E. lindenii*, measured by seed set after a single visit to the flower at Serra do Órgãos National Park, southeastern Brazil.

1 **Table 3:**

2 **The pollination efficacy of hummingbirds was higher than efficacy of large bees in**

3 ***Edmundoa lindenii.***

4 ANOVA results on the pollination efficacy of functional groups of pollinators of *E. lindenii*,

5 measured by seed set after a single visit to the flower at Serra do Órgãos National Park,

6 southeastern Brazil.

Effects	DF	F	p
Reproductive event (year)	2	0.15	0.860
Functional group of pollinators	1	71.07	**<0.001**
Reproductive event : functional group	1	2.60	0.078

The reproductive events and the functional groups of flower visitors of *Edmundoa lindenii* had an effect on the relative frequency of visits.

ANOVA results on the relative frequency of floral visits by functional groups during the reproductive events of 2015, 2016, 2017 and 2018 at Serra do Órgãos National Park, southeastern Brazil.

1 **Table 4:**

2 **The reproductive events and the functional groups of flower visitors of *Edmundoa lindenii***

3 **had an effect on the relative frequency of visits.**

4 ANOVA results on the relative frequency of floral visits by functional groups during the

5 reproductive events of 2015, 2016, 2017 and 2018 at Serra do Órgãos National Park,

6 southeastern Brazil.

Effects	DF	Deviance	P (> Chi)
Reproductive events (year)	3	0.020	0.999
Functional group of floral visitors	2	**26.392**	**<0.001**
Reproductive events : functional group	6	**244.562**	**<0.001**