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1 **Factors influencing the cycles of foraging activity and inactivity in colonies of the**  
2 **leaf-cutting ant *Atta sexdens* (Linnaeus) in Eucalyptus plantations**

3

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16

17 **Short running title:** Foraging inactivity in leaf-cutting ant colonies

18

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25 **ABSTRACT**

26 Foraging in leafcutter ants alternates between periods of inactivity and activity that extend  
27 over several days or weeks. Many factors could influence this alternation, with a  
28 preponderance of environmental factors, such as temperature, humidity, and rain.  
29 Moreover, ant colony size and the management practices used for their control could also  
30 modify the cycles of activity/inactivity. We surveyed the foraging activity of the nests of  
31 the leaf-cutting ant *Atta sexdens* (Linnaeus) in Eucalyptus plantations and evaluated the  
32 relative importance on foraging activity of temperature, past possible immediate  
33 experience in foraging, and use of machinery. Twenty transects with an average of 6  
34 colonies in each were selected. Each nest was checked for foraging activity once in  
35 different months of the year and we investigated whether foraging inactivity was  
36 influenced by nest size, temperature, number of favorable hours for foraging on the day  
37 preceding the evaluation, and the use of agricultural machinery. The management  
38 practices of *A. sexdens* nests in Eucalyptus plantations had no influence on the occurrence  
39 of foraging inactivity, contrary to nest size and possible past immediate experience in  
40 foraging. The probability of foraging inactivity decreased by about 23% for each 10m<sup>2</sup>  
41 increment in the nest size. A nest was also more likely to be active on a certain day if the  
42 conditions for foraging activity had been favorable on the preceding day. Leaf-cutting  
43 ants are one of the main herbivores in Brazil, often reaching pest status in several crops.  
44 Our results indicate that the efficiency of their control in Eucalyptus plantations could be  
45 increased by implementing control strategies several times a year and by increasing the  
46 number of bait applications in areas with a high density of small nests (less than 20 m<sup>2</sup>),  
47 as these areas are more prone to inactivity and thus may lead to increased control failures.

48

49 **Keywords:** colony size, forest plantations, machinery, rain, temperature.

## 50 INTRODUCTION

51 Leaf-cutting ants of the genus *Atta* and *Acromyrmex* (Hymenoptera: Formicidae)  
52 count among one of the most fascinating insects in the world. They practice a sort of  
53 primitive agriculture: they cut and collect vegetation in the environment and bring it back  
54 to their nest to feed a symbiotic fungus as their main source of food (Holldobler & Wilson,  
55 2010). These ants form huge colonies of several hundred thousand individuals and their  
56 underground nests sometimes occupy more than one hundred cubic meters (Moreira et  
57 al., 2004). Because of their defoliation action, they cause considerable damage to food  
58 and industrial crops (e.g. sugarcane, Eucalyptus) and they are thus considered the main  
59 herbivore in the Neotropical region and a major pest for cultivated plants (Della Lucia et  
60 al. 2014; Leal et al. 2014; Zanetti et al. 2014).

61 The foraging activity of leaf-cutting ants follows a circadian rhythm (Lewis et al.  
62 1974; Rockwood 1975; Caldato et al. 2016) influenced by several external factors, such  
63 as air temperature and humidity (Giesel et al. 2013; Tizón et al. 2014; Bustamante et al.  
64 2020), rain (Farji-Brener et al., 2018), wind (Alma et al., 2016), and natural enemies such  
65 as parasitoids (Yackulic and Lewis 2007; Guillade and Folgarait 2015). Leaf-cutter ants  
66 also show a high plasticity in their activity rhythm. Therefore, the same species can have  
67 different patterns of foraging activity depending on the environment in which it lives, e.g.  
68 forests, savannas, or pastures (Kost et al. 2005; Urbas et al. 2007; Wirth et al. 2007).  
69 However, the foraging activity of leaf-cutting ants alternates between periods of activity  
70 and inactivity of several days, or even weeks, and the origin of this phenomenon remains  
71 unknown. The fact that these periods are not necessarily synchronized between the  
72 colonies located in the same area is particularly intriguing. In cultivated areas such as  
73 pastures or planted forests, where leaf-cutting ants are common, human disturbances  
74 could also play a role in determining these cycles of activity/inactivity. In planted forests,  
75 for example, tractors are used several times a year to manage the soil, treat weeds, and  
76 control leaf-cutting ants. However, the effects of this disturbance on the foraging of leaf-  
77 cutting ants have so far not been documented.

78 In this paper, we tested the hypothesis that environmental and anthropic factors  
79 could be responsible for alternating foraging activity/inactivity in leaf-cutting ants. First,  
80 we hypothesize that leaf-cutting ants would be less likely to be inactive on a given day if  
81 this day was preceded by a day with a long period of rainfall (Farji-Brener et al., 2018),  
82 or a day during which the values of air temperature and humidity were not favorable for

83 foraging activity (Fowler & Robinson, 1979; Giesel et al., 2013). Second, we hypothesize  
84 that in cultivated areas such as Eucalyptus plantations, anthropic factors like the use of  
85 agricultural machinery could also influence the foraging activity of the colonies, for  
86 example through a change in the physical structure of the nests (Leal et al., 2014). Indeed,  
87 leaf-cutting ants build extensive networks of underground tunnels (Moreira et al., 2004)  
88 that are essential for maintaining an internal microclimate inside their nest suitable for  
89 the cultivation of their symbiotic fungus (Bollazzi & Roces 2007; Bollazzi et al., 2008).  
90 The use of agricultural machinery in cultivated areas is likely to damage the structure of  
91 these tunnels and, therefore, to compromise the integrity of the fungus garden. Hence,  
92 after a nest has been damaged, more workers may be relocated to excavation tasks (Farji-  
93 Brener & Tadey, 2012) at the expense of foraging, leading to a sharp decrease or a  
94 complete stop of foraging activity during several days. Identifying the factors responsible  
95 for the cycles of foraging activity/inactivity could help to promote better control strategies  
96 that minimize waste and environmental contamination and better predict the damages  
97 caused by leaf-cutting ant colonies in cultivated areas.

98

## 99 **MATERIALS AND METHODS**

### 100 *Studied site*

101 The leaf-cutting ant colonies studied were located in six different stands of  
102 Eucalyptus plantations in an area of Atlantic Forest situated in the Eastern part of the state  
103 of Minas Gerais, Brazil (Ipatinga -MG). The experiments were carried out in areas with  
104 more cohesive and clayey soils. No stands had been chemically or mechanically treated  
105 for one year at the start of the study. Each of the six stands was surveyed only once  
106 between June 2020 and May 2021.

107

### 108 *Factors influencing foraging inactivity*

109 Twenty 12 x 100m transects were delimited in each stand of Eucalyptus plantation  
110 and the number of *Atta sexdens* (Linnaeus) nests found inside these transects was counted.  
111 Each nest was georeferenced and its size was measured by the larger rectangular area,  
112 including all the mounds of loose excavated soil above the nests (Grandeza et al., 1999).

113 The foraging activity of all nests within the 6 stands was evaluated once between  
114 June 2020 and May 2021 in one of the following months: June 2020, July 2020,  
115 September 2020, December 2020, January 2021, May 2021. We used the methodology  
116 proposed by Fowler et al. (1993) which consists in placing plastic baits (length: 1 cm)

117 coated with a powder made from citric pulp close to the nests and to evaluate whether  
118 these baits are then rejected with excavated soil on the mound of loose soil above the nest.  
119 A colony was considered active in our study if ants rejected the baits within 24 hours,  
120 otherwise it was considered inactive. The temperature, humidity, and rainfall were  
121 obtained from the meteorological data of the closest weather station to the surveyed stands  
122 (Figure 1). These data were used to calculate the number of hours in which the  
123 meteorological conditions were favorable for the foraging activity of *A. sexdens* colonies  
124 on the day preceding our evaluation. We reasoned that foraging activity was more likely  
125 to be observed on the day of evaluation if the meteorological conditions on the previous  
126 day had been favorable for foraging. Based on a previous survey of the foraging activity  
127 of *A. sexdens* colonies (Abreu 2015; Bustamante and Amarillo-Suárez 2019), we  
128 considered that foraging activity was more likely to occur during an hour if the  
129 temperature was in the range of 18 - 23°C and if no rainfall had occurred. Our previous  
130 activity survey shows that in these conditions the probability of finding ants carrying  
131 vegetation back to their nest on foraging trails reaches at least 25%, half the maximum  
132 value that we observed.

133 A logistic regression was used to investigate the influence of nest size, the impact  
134 mean temperature on the day of the survey, and the number of favorable hours for  
135 foraging activity on the day preceding the survey of foraging activity, on the probability  
136 for the colonies of being inactive. The analysis did not consider relative humidity because  
137 it is highly correlated with temperature. We also tested the interaction between nest size  
138 and temperature because we reasoned that the probability of being inactive could vary  
139 differently with temperature in colonies of different sizes, with small colonies being more  
140 likely to be inactive at high temperatures than large ones. Worker size polymorphism is  
141 known to vary with colony age, with large colonies having on average larger workers and  
142 a higher size polymorphism than small ones (Wilson, 1980). Since there is a link between  
143 worker size and thermotolerance in leaf-cutting ants (Ribeiro et al., 2012; Bouchebti et  
144 al., 2014; Baudier & O'Donnell 2020), large colonies could thus be active for a larger  
145 temperature range than small colonies.

146

#### 147 ***Impact of tractor passage on foraging activity***

148 Twenty additional transects were delimited in the stands of the Eucalyptus  
149 plantation that was used to evaluate foraging activity. The colonies of *A. sexdens* found  
150 in these transects were counted and georeferenced and their size was estimated by the

151 area covered by the mounds of loose soil over the nest. The foraging activity of each nest  
152 was then assessed in the same way as described above. Then, after 24 hours, a tractor  
153 weighing 3500kg drove over the whole area of the transects and all leaf-cutting ant nests.  
154 Nest activity was then reassessed 24 hours after the tractor passage using the previously  
155 described method. No implement was attached to the tractor, only its weight was  
156 considered to damage the colonies.

157 The effect of the tractor on the probability of a nest being inactive was tested with  
158 a binomial GLMM. The chambers of small nests of *A. sexdens* are much closer to the soil  
159 surface than those of large ones (Camargo et al., 2016). Therefore, small nests are  
160 probably more likely to be affected by the passage of the tractor than large ones and the  
161 probability of being inactive may thus vary differently before and after the passage of the  
162 tractor for nests of different sizes. Consequently, we included the following independent  
163 variables in the model: tractor passage (with two levels: control – before the passage of  
164 the tractor, treatment – after the passage of the tractor), nest size, and the interaction  
165 between these two variables. The nest identity was included as a random variable in the  
166 model.

167 All analyses were performed and graphics generated with R 3.4.1. The package  
168 *ggplot2* (Wickam et al., 2022) was used to create the graphics. The package *lme4* was  
169 used to perform the GLM and GLMM (Bates et al., 2015) and the package *DHARMA*  
170 (Harting & Lohse, 2022) to check the quality of the statistical models. The significance of  
171 each model variable was assessed by using the R *drop1()* function which deletes each  
172 single term from the full model and then compares the change in fit of the model before  
173 and after deletion with a  $\chi^2$  test.

174

## 175 **RESULTS**

### 176 ***Characterization of Leaf-Cutter Ant Infestation in Experimental Plots***

177 A total of 579 *A. sexdens* nests were surveyed in the study. The majority of nests were  
178 relatively small, with those under 10 m<sup>2</sup> being the most common. In contrast, larger nests,  
179 exceeding 60 m<sup>2</sup>, were less frequently encountered (Figure 2a) which concurs with the  
180 typical leaf-cutting ant nest size distribution (Caldeira et al. 2005). Most of the transects  
181 showed an average of 3 to 6 nests (Figure 2b), reflecting a moderate nest density across  
182 the study area. High infestation levels, defined as transects with more than 9 nests, were  
183 rare and difficult to identify.

184

185 ***Evaluation of foraging inactivity***

186 The mean daily air temperature on the day of evaluation had no statistically  
187 significant influence on the occurrence of foraging inactivity (Table 1). On the other hand,  
188 nest size slightly influenced the probability of foraging inactivity (Table 1): independent  
189 of the number of favorable hours for foraging and of daily average temperature, small  
190 nests were more likely to be inactive than large ones (Figure3). The number of favorable  
191 hours for foraging on the day preceding the evaluation had also a statistically significant  
192 effect on the occurrence of foraging inactivity (Table 1): independent of nest size and  
193 daily average temperature, the colonies were more likely to be inactive if the number of  
194 favorable hours was small (Figure 4).

195

196 ***Impact of tractor passage on foraging activity***

197 There was no interaction between the size of the colonies and the passage of the  
198 tractor over the nests. In other words, the damages caused by the tractor had no impact  
199 on the occurrence of foraging inactivity, regardless of colony size. Moreover, there was  
200 no statistically significant effect of the damages caused by the tractor or the size of the  
201 nests on the occurrence of inactivity of the colonies (Table 2).

202

203 **DISCUSSION**

204 Leaf-cutting ants are peculiar because, contrary to most tropical insects, they are  
205 characterized by an alternation of foraging activity/inactivity spanning over several days  
206 or weeks. In species of ants living in high latitudes, activity is strongly dependent on  
207 seasonal changes in meteorological conditions, and ants can remain inactive for several  
208 months in the winter period (Jacquier et al., 2021). However, inactivity periods in leaf-  
209 cutting ants are shorter and can be observed even when the meteorological conditions are  
210 relatively stable. In this work, we found that leaf-cutting ants foraging inactivity is  
211 unrelated to temperature. On the other hand, we found that past immediate experience  
212 can affect foraging activity as well as factors specific to the colonies, such as their size.  
213 Finally, we show that the use of machinery to control leaf-cutting ants in eucalyptus  
214 forests does not affect ant foraging activity, at least in our studied region.

215 Temperature is a limiting factor for foraging in leaf-cutting ants, with colony  
216 foraging activity generally being observed only within a given range of ambient  
217 temperatures (Giesel et al., 2013; Caldato et al., 2016; Baudier & O'Donnell 2020; Welch  
218 et al., 2020). Temperature changes can modulate foraging activity in different ways, e.g.

219 the intensity of worker flows on foraging trails (Giesel et al., 2013), the speed and size of  
220 foraging workers (Burd, 1996; Ramirez-Olier et al., 2022), and the proportion of workers  
221 carrying leaf fragments and thus the rate of vegetation biomass carried to the nest  
222 (Endringer et al., 2012). Nevertheless, we found no influence of ambient temperature on  
223 the foraging activity of *A. sexdens* colonies in the present study. *A. sexdens* is widely  
224 distributed in Brazil and it is characterized by a high thermotolerance. This should allow  
225 this species to forage in a wide range of temperatures in rural and urban areas (Angilletta  
226 et al., 2007; Roeder et al., 2021). Nevertheless, the range of ambient temperatures to  
227 which the colonies were exposed during our survey may have been too narrow to  
228 significantly impact foraging activity.

229         The greater probability of small colonies (size < 20 m<sup>2</sup>) of being more inactive  
230 than large ones (size > 60 m<sup>2</sup>) could be linked to the size of the workers in the colony.  
231 Leafcutter ants show strong polymorphism, and the size of workers varies according to  
232 age and colony size (Hölldobler & Wilson, 2010). The average size of the workers in  
233 young colonies is smaller than in old ones and there is less variation in the size of foraging  
234 workers (Hölldobler & Wilson, 1990). This may restrict the range of temperatures at  
235 which the colonies can forage. In fact, large workers are more tolerant to low or high  
236 temperatures than small ones, because of their lower surface/volume ratio (Bouchebti et  
237 al., 2014; Bujan et al., 2016; Perl & Niven, 2018; Packard, 2020). Therefore, small  
238 colonies' activity/inactivity should be more dependent on ambient conditions than large  
239 colonies. Another factor that could affect the foraging activity of small nests is  
240 competition. The density of *A. sexdens* nests in Eucalyptus plantations can reach a high  
241 level and the nest distribution is generally aggregated (Camargo, 2008; Souza et al.,  
242 2011). This may increase the effect of competition. Big colonies are also likely to have  
243 higher competitive advantages than small ones because their division of labor is more  
244 efficient and they are thus able to handle a high rate of leaf fragments reaching their nests  
245 (Wilson, 1980). In addition, the larger workers of big colonies can collect more plant  
246 biomass *per capita* (Bouchebti et al., 2015) and are more efficient at defending the colony  
247 than smaller workers from young colonies. Furthermore, small colonies have a smaller  
248 fungus garden volume than large colonies and are, therefore, more vulnerable to  
249 starvation during prolonged periods of adverse weather conditions. This could explain the  
250 high mortality rate observed in colonies at an early stage of development (Zanetti et al.,  
251 2014).

252           The number of hours favorable for foraging on the day preceding the survey had  
253 a statistically significant effect on the probability of a nest being inactive: colonies of any  
254 size were less likely to be inactive when the number of hours favorable for foraging on  
255 the day preceding the survey was low. A nest was thus more likely to be active on a certain  
256 day if the conditions for foraging activity had been favorable on the preceding day.  
257 Therefore, ants did not stop foraging because of a possible accumulation of vegetation  
258 biomass in their nest. The nests used in this survey probably had many chambers with  
259 fungus gardens and would, therefore, be capable of incorporating a large volume of  
260 vegetation. For example, a two-year old 56 m<sup>2</sup> nest can contain around 390 chambers with  
261 a fungus garden (Autori, 1942; Swanson et al., 2019).

262           We found that the passage of the tractor over the nests had no impact on the  
263 colonies' inactivity. This could be because large *A. sexdens* nests are quite deep. Even in  
264 young colonies, the depth of the chambers can range from 10 to 30 cm below the soil  
265 surface (Camargo et al., 2016). Therefore, the weight of the tractor and the pressure it  
266 exerted on the ground were probably insufficient to cause significant-damage to the nests.  
267 Moreover, the soils of our survey areas have a high content of clay and organic matter  
268 and thus are less likely to lose their physical structure than other types of soil when  
269 subjected to strong pressure (Bronick & Lal 2005). This finding is particularly relevant,  
270 as tractor use is common in forestry operations, especially for fertilization, weed and leaf-  
271 cutting ant control. The fact that the tractor's wheel pressure does not induce inactivity in  
272 the colonies suggests that the use of machinery does not lead to a decrease in control  
273 effectiveness.

274           Leaf-cutting ants show a high capacity to establish their nests in disturbed  
275 conditions, even in areas with high use of agricultural machinery. This explains why they  
276 reach a pest status in many crops (Robinson & Fowler, 1982; Zanetti et al., 2014).  
277 Chemical control with granulated formicide baits is often the only method to control their  
278 population. Therefore, knowledge of the factors that influence the period and duration of  
279 these ants' foraging inactivity is essential for their management. This knowledge would  
280 help to adjust the timing of application of these baits, improving control efficiency and  
281 reducing their effects on non-target insects. Our study shows that applying formicide baits  
282 twice or more a year in areas with a high density of nests smaller than 20 m<sup>2</sup> may be  
283 particularly beneficial. In fact, these areas are more likely to experience periods of  
284 inactivity, during which the effectiveness of control measures may be reduced, leading to  
285 the persistence of the leaf-cutter ant population. Repeated interventions in such areas may

286 help to mitigate these gaps in control, improving overall management results. However,  
287 it is also essential to consider the memory mechanisms of leaf-cutting ants (Falibene et  
288 al., 2015; Saverschek et al., 2010; Saverschek & Roces, 2011), as they cease to transport  
289 baits when they have associated the presence of attractants with mortality within the  
290 colony, which may compromise long-term control efficacy. Therefore, it is crucial to  
291 adjust correctly the interval between chemical bait applications to ensure control efficacy.

292

### 293 **Competing Interests**

294 The authors declare that they have no conflicts of interest to this work.

### 295 **Data availability**

296 The datasets used during the current study is available from the corresponding author on  
297 reasonable request.

### 298 **Ethics declarations**

299 The authors declare that the work complies with the requested ethical standards.

### 300 **Study complies**

301 All study procedures involving plants in the present study comply with relevant  
302 institutional, national, and international guidelines and legislation.

### 303 **Author Contributions**

304 JJS and JVSJ collected and analysed the data, prepared figures and tables. VF conceived  
305 and designed the experiments, analysed the data. CSA secured funding. RZ conceived  
306 and designed the experiments, acquired funds, analysed the data. All authors authored  
307 and reviewed drafts of the article, read and approved the final draft.

308

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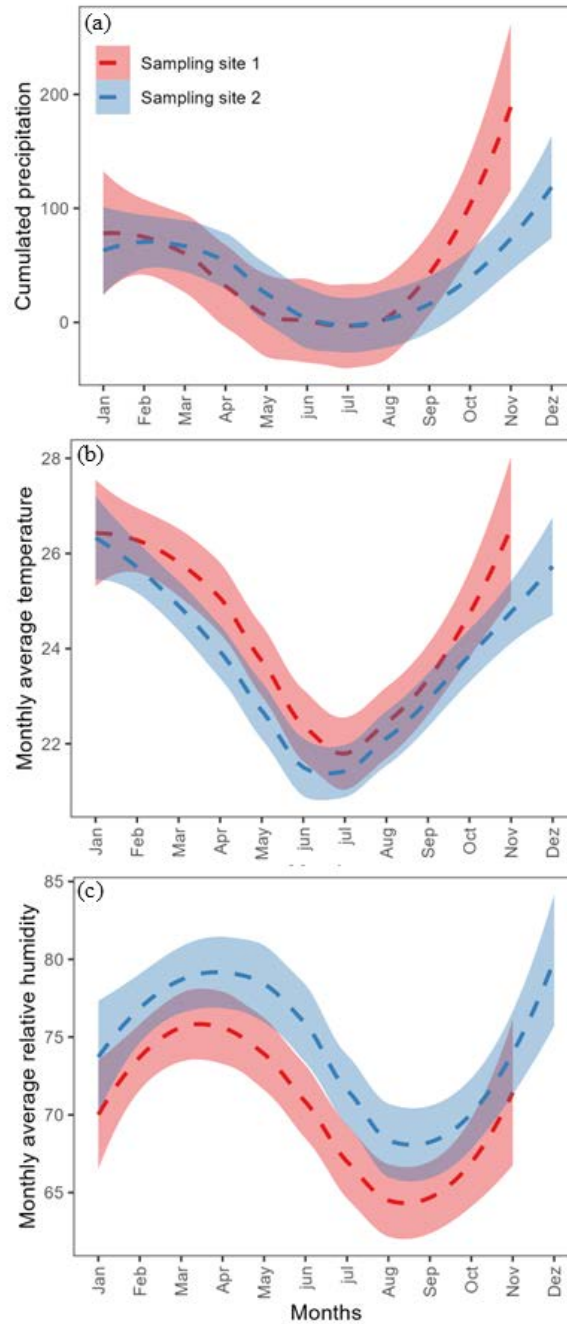
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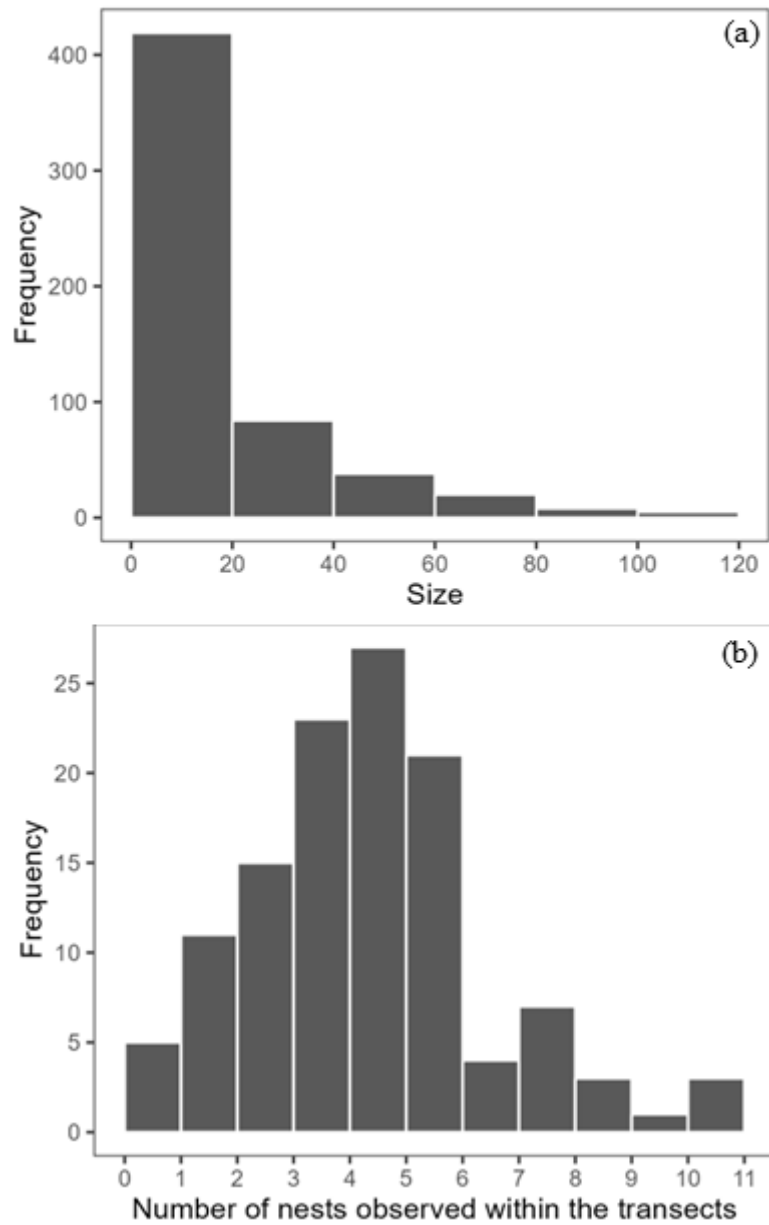
493 **FIGURES**

494



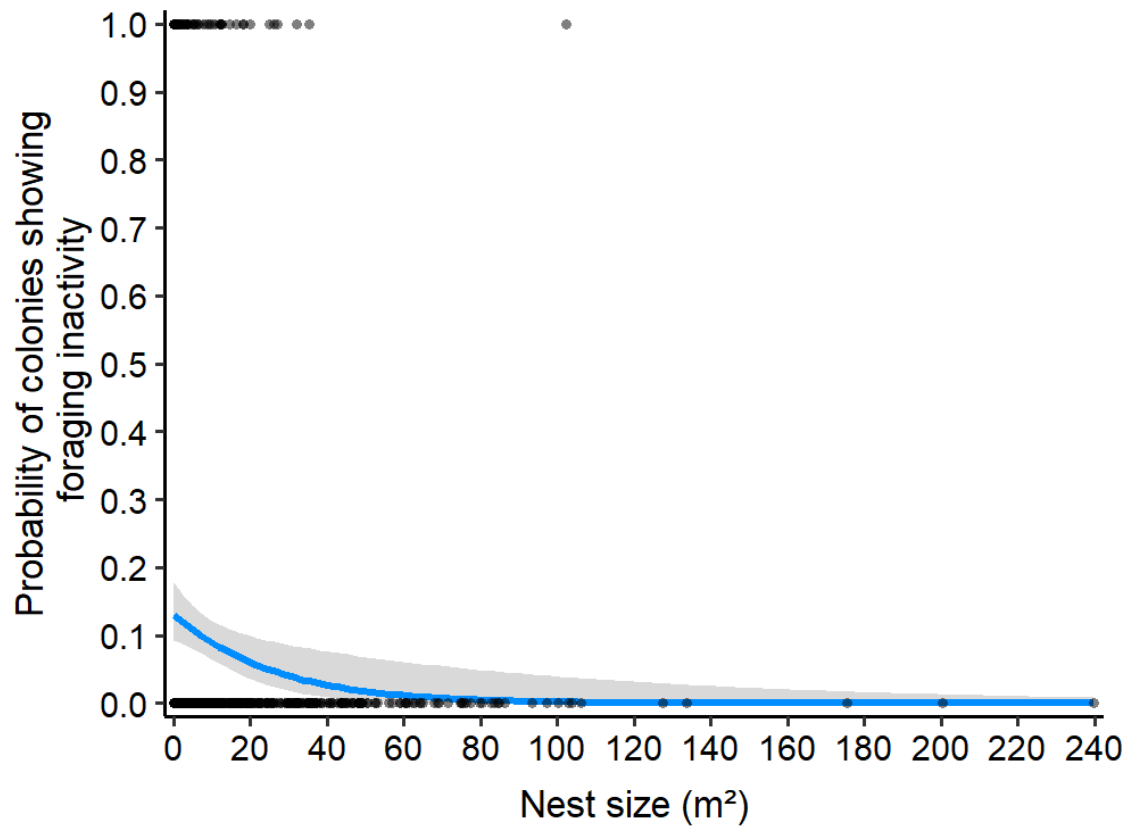
495

496 Figure 1. Climate patterns recorded over three successive years (2019-2021), including the two years of the  
 497 survey (2020-2021), calculated from data of the nearest meteorological station to the two collection sites.  
 498 (a) Monthly average cumulated precipitation in mm. (b) Monthly average temperature in °C. (c) Monthly  
 499 average relative humidity in %. "Sampling site 1" refers to collections conducted in June and July 2020,  
 500 while "Sampling site 2" to those conducted in September and December 2020, and in January and May  
 501 2021. The shaded areas represent the confidence interval of the mean values. The data presented provide a  
 502 comprehensive overview of the climatic conditions in the regions where the data were collected, which is  
 503 critical for understanding their potential influence on the studied ecological processes.



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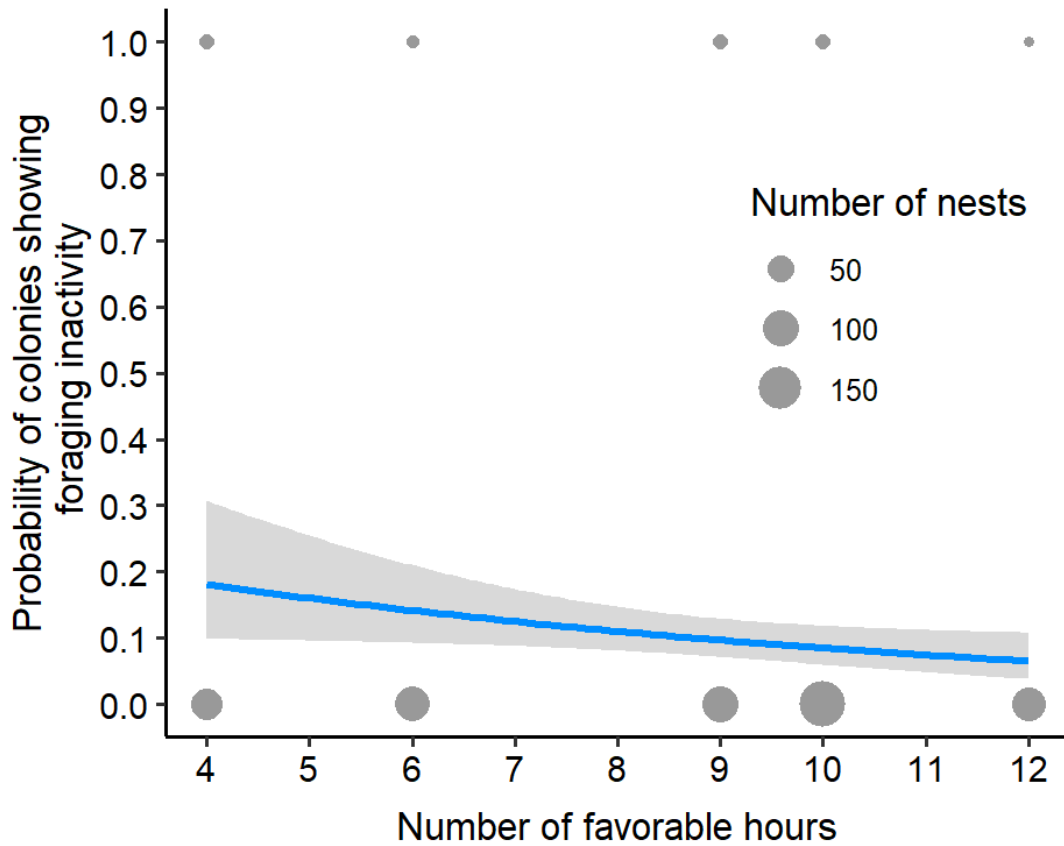
505 Figure 2: Frequency histogram of *Atta sexdens* nest sizes observed across all transects (a) and average  
 506 number of nests per transect (b). The majority of nests were smaller than 10 m<sup>2</sup>, with few nests  
 507 exceeding 60 m<sup>2</sup>. Most transects contained between 4 and 6 nests, reflecting a moderate nest density.  
 508 High infestation levels (more than 9 nests per transect) were rarely observed.



509

510 **Figure 3.** Probability of *Atta sexdens* colonies showing foraging inactivity as a function of nest size. Small  
 511 nests of *Atta sexdens* have a higher probability of showing foraging inactivity than large ones. The figure  
 512 shows the predictions of the probability as a function of nest size for the mean temperature (22.8°) and the  
 513 mean number of hours favorable for foraging on the day preceding the evaluation of foraging activity, i.e.  
 514 9 hours. The grey area represents the confidence interval of the predictions. N = 579 nests.

515



517

518 **Figure 4.** Probability of *Atta sexdens* colonies showing foraging inactivity as a function of the number of  
 519 hours favorable for foraging on the day preceding the evaluation of foraging activity. The figure shows the  
 520 predictions of the probability as a function of the number of favorable hours for foraging for the mean  
 521 temperature (22.8°) and the mean size of the nests in the sample, i.e. 17.44m<sup>2</sup>. The grey area shows the  
 522 confidence interval of the predictions. The size of the points is proportional to the number of nests with the  
 523 corresponding value.  $N=579$  nests.

524 **TABLES**

525 Table 1. Influence of nest size, temperature and the number of hours favorable for foraging on the day  
 526 preceding the evaluation of foraging activity, on the occurrence of inactivity in *A. sexdens* nests.

Variables	df	Deviance <sup>a</sup>	$\chi^2$	<i>P</i> <sup>b</sup>
Nest size	1	366.64	4.00	<b>0.0454</b>
Mean temperature	1	363.70	1.07	0.3013
Number favorable hours	1	368.58	5.95	<b>0.0147</b>
Mean temperature x Nest size	1	365.90	3.27	0.0705
Number favorable hours x Nest size	1	365.18	2.55	0.1108

527 <sup>a</sup> *GLM* (family: binomial; link: logit; response: inactive [=1], active [=0]). The results show the  
 528 change in fit in the model when each single term of the full model is deleted in turn (type III Anova).

529 <sup>b</sup> *p*-values lower than the significance threshold of 0.05 are indicated in bold.

530 Table 2. Influence of forest management practice on the occurrence of inactivity in *Atta*  
 531 *sexdens* nests

Variables	df	$\chi^2$ <sup>a</sup>	<i>P</i> <sup>b</sup>
Nest size	1	3.6429	0.0563
Tractor passage	1	2.1640	0.1413
Nest size x Tractor passage	1	2.4445	0.1179

532 <sup>a</sup> GLMM (family: binomial; link: logit; response: inactive [=1], active [=0]). The results show  
 533 the change in fit in the model when each single term of the full model is deleted in turn (type III  
 534 Anova).

535 <sup>b</sup> *p*-values lower than the significance threshold of 0.05 are indicated in bold.