

1 Article

2 **Nest site selection during colony relocation in**  
3 **Yucatan Peninsula populations of the ponerine ant**  
4 ***Neoponera villosa* (Hymenoptera: Formicidae)**

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13 Received: 15 January 2020; Accepted: date; Published: date

14 **Abstract:** In the Yucatan Peninsula, the ponerine ant *Neoponera villosa* nests almost exclusively in  
15 tank bromeliads, *Aechmea bracteata*. We aimed at determining factors influencing nest-site selection  
16 during nest relocation regularly promoted by hurricanes in this area. Using ants with and without  
17 previous experience of *Ae. bracteata*, we tested their preference for refuges consisting of *Ae. bracteata*  
18 leaves over two other bromeliads, *Ae. bromeliifolia* and *Ananas comosus*. We further evaluated  
19 bromeliad-associated traits that might influence nest-site selection (form, size). Workers with and  
20 without previous contact with *Ae. bracteata* significantly preferred this species over others,  
21 suggesting the existence of an innate attraction to this bromeliad. However, preference was not  
22 influenced by previous contact with *Ae. bracteata*: workers easily discriminated between shelters of  
23 *Ae. bracteata* and *A. comosus*, but not those of the closely related *Ae. bromeliifolia*. In marked contrast,  
24 ants discriminated between similar sized *Ae. bracteata* and *Ae. bromeliifolia* plants, suggesting that  
25 chemical cues and plant structure play an important role. Size was also significant as they selected  
26 the largest plant when provided two dissimilar *Ae. bracteata* plants. Nest-site selection by *N. villosa*  
27 workers seems to depend on both innate preferences but familiarization with plant stimuli is not  
28 excluded.

29 **Keywords:** *Aechmea bracteata*; bromeliad; Ponerinae; tandem running; colony relocation; adaptation.

30

31 **1. Introduction**

32 Many species of social hymenoptera frequently move to new nests sites although emigration  
33 presents significant challenges and risks [1,2], and often implies a fitness cost [3,4]. Colony relocation  
34 is a common phenomenon in ants [1,5]. Some ant species move their nests as part of their life history  
35 (e.g. army ants), but the majority do so in response to numerous biotic and abiotic factors, including  
36 microclimate fluctuation [6–8], physical disturbance [9,10], intra and interspecific competition  
37 [1,4,11–13], resource availability [14,15], and predator or parasite pressure [5,12,14,16]. Arboreal ants  
38 are particularly prone to move their colonies from one site to another [1] as occurs commonly in the  
39 Neotropical ponerine ant, *Neoponera villosa* (Fabricius) (Hymenoptera: Formicidae) [17]

40 *Neoponera villosa* is a generalist arboreal predatory ant [18–20] with a wide geographical  
41 distribution, from Texas to Argentina [21]. This species occurs both in wet and dry forests [22], and  
42 is an opportunistic cavity-breeder that nests in dead and live trees, and in bromeliads [17,23,24]. In  
43 the southern part of the Yucatan Peninsula, Mexico, *N. villosa* nests mainly in the epiphytic bromeliad

44 *Aechmea bracteata* (Sw.) Griseb [23,25,26] although other species of *Aechmea* with the same type of  
45 growth are available in this area [27]. Workers measure 12 - 13 mm [28] and colonies nesting in *Ae.*  
46 *bracteata* contain  $97.8 \pm 7.9$  workers (mean  $\pm$  SEM,  $n = 82$ ; range: 3 - 322) [29]. *Aechmea bracteata* is a  
47 "phytotelm tank" type bromeliad; mature plants present a waterproof central cavity suitable for  
48 housing ants [23,26]; large groups of shoots at different stages of maturity develop from a rhizome  
49 [30]. This bromeliad is characteristic of the inundated forest of the Sian Ka'an Biosphere Reserve  
50 where clusters are found established at a mean height of 1.3 m [26]. Like other large tank bromeliads,  
51 *Ae. bracteata* individuals offer permanent shelter to a wide diversity of organisms, both specialists  
52 and opportunists [23,26, 31,32], and during extreme flooding and other climatic events they constitute  
53 ecological refuges for many other ground-dwelling arthropods [33]. As with most myrmecophytes,  
54 *Ae. bracteata* can be associated with several ant species, including *N. villosa* [26]; however, it does not  
55 depend on ants for its germination [34].

56 *Neoponera villosa* is not an obligate inhabitant of myrmecophytes; however, in the southern  
57 region of the Yucatan Peninsula, this ant uses the tank-bromeliad as a nest throughout the year,  
58 displaying a very marked local specialization [25]. There is little knowledge regarding the evolution  
59 of host-plant specialization between plants and ants in facultative associations. In the case of ants  
60 that nest in specific plants, it has been shown that host plant recognition is primarily based on two  
61 factors: an innate (genetically determined) attraction towards certain plants rather than others, and  
62 the influence of the environment during development and early adult life (pre-imaginal learning and  
63 conditioning through contact with the host plant during larval life and the first days of adult life),  
64 that may even supplant a genetically determined attraction or deterrence [25,35-37]. For instance, the  
65 African arboreal ants *Tetramorium aculeatum* (Mayr) (Myrmicinae) and *Oecophylla longinoda* (Latreille)  
66 (Formicinae) present a familiarization process (early learning) that can replace the innate attraction  
67 of both species [36,37]. This learning only takes place during the neonatal stage, a sensitive period  
68 after which the influence of the environment ceases [see 35]. Attraction to *Ae. bracteata* by alate queens  
69 (gynes) and young *N. villosa* workers (nurses) has been studied in the context of new colony  
70 foundation by foundress females [25]. Gynes from colonies nesting in *Ae. bracteata* are attracted to  
71 this bromeliad, a preference that appears to be learned during the larval stage. This pre-imaginal  
72 learning may be further strengthened at the beginning of the imago life, causing local fidelity toward  
73 *Ae. bracteata* over other available species [25]. However, nest-site selection in *N. villosa* has not been  
74 studied in the context of nest-relocation, a distributed, nonhierarchical decision-making process  
75 which is performed by several scouts who find potential nest sites. Informed scouts lead nestmates  
76 to the chosen new nest sites through tandem running, with only one nest mate being recruited at a  
77 time. The new nest site is defined by a quorum sensing mechanism, i.e. when more ants are present  
78 at one of the alternatives [5].

79 The Yucatan Peninsula has been identified as a region that is affected by hurricanes and  
80 droughts [38], which can result in bromeliads dislodging from their host tree and falling to the  
81 ground, thus requiring complete ant colonies to relocate. For cavity-nesting species such as *N. villosa*,  
82 there is only a limited number of potential nest sites that can meet the requirements of a mature  
83 colony. Furthermore, nesting sites are competitively searched for by other species, specifically  
84 *Dolichoderus bispinosus* (Olivier) and *Nasutitermes* sp. [23,26]. In most cases, scouts encounter various  
85 candidate shelters and have to decide which is the most suitable. Some characteristics of the potential  
86 nest site, in particular the size of the nesting cavity, can constrain colony growth [39-43] and this  
87 factor is expected to influence nest-site choice in *N. villosa* [23]. Furthermore, some ant species can  
88 assess nest-site suitability through various physical characteristics such as darkness, cavity height,  
89 entrance width and configuration [44,45]. However, with regard to *N. villosa*, the stimuli which  
90 intervene during nest-site selection have not been identified. In various species of ants that establish  
91 obligate interactions with plants, it has been demonstrated that host plant recognition is primarily  
92 based on chemical cues that attract foundresses [46-51]. However, plant height, nest-site geometry  
93 or clear areas around trees that provide information on the size of the potential nest candidate or on  
94 its protective potential, are used by various animal species as cues during nest-site selection [42,52-  
95 55] and may also play an important role during nest relocation in *N. villosa*.

96 In the present study, we performed different experiments (two-choice bioassays) to determine  
97 how *N. villosa* workers select a nest-site in the eventuality of nest relocation. Because rearing workers  
98 from egg to adult was not feasible, we took advantage of the fact that *N. villosa* nests almost  
99 exclusively in cavities of live trees in northern Yucatan where *Ae. bracteata* is rare, to investigate nest-  
100 site selection of *N. villosa* workers without previous contact with this bromeliad. Our research  
101 addressed the following questions: 1) Do *N. villosa* workers have an innate preference for *Ae.*  
102 *bracteata*? 2) Is the preference modulated by the pre-imaginal or neonatal ant experience linked to the  
103 origin of the colony (workers with or without previous contact with *Ae. bracteata*)? 3) Are the  
104 recognition and localization of *Ae. bracteata* regulated by chemical stimuli? 4) Does *Ae. bracteata* size  
105 influence nest-site selection?

## 106 2. Materials and Methods

### 107 2.1. Ant collection and identification

108 Ants in bromeliads were collected in five sites in the southern part of the Yucatan Peninsula:  
109 Ejido Blasillo (18°7'37.98"N, 89°20'20.93"W, 261 m.a.s.l.), Nuevo Becal (18°36'39.36"N, 89°16'15.54"W,  
110 239 m.a.s.l.) and Zoh-Laguna (18°35'11.61"N, 89°25'4.67"W, 257 m a.s.l.) in Campeche; Kohunlich  
111 (18°25'31.08"N, 88°48'9.89"W, 143 m.a.s.l.) and Sian Ka'an Biosphere Reserve (19°41'56.17"N,  
112 87°50'18.31"W, 18 m.a.s.l.) in Quintana Roo. Ants nesting in tree cavities (mainly *Lysiloma latisiliquum*  
113 (L.) Benth., *Caesalpinia gaumeri* (Britton and Rose) Greenm., and *Leucaena leucocephala* (Lam.) de Wit  
114 (Fabaceae), and *Bursera simaruba* (L.) Sarg. (Burseraceae)) were collected essentially in Cuxtal  
115 Ecological Reserve (20°51'46.58"N, 89°36'40.68"W, 17 m.a.s.l.) in Yucatan, in the northern part of the  
116 Peninsula, but a few were collected in the south, in Chetumal (18°32'37.90"N, 88°15'46.38"W, 10  
117 m.a.s.l.) in Quintana Roo. In the latter two sites, *Ae. bracteata* is rare or absent. Each epiphyte was cut  
118 off from the supporting branch, dismantled leaf-by-leaf in plastic bins coated with Fluon (Whitford  
119 GmbH, Diez, Germany), and all *N. villosa* ants and their brood were collected. Both ants from  
120 epiphytes and those from tree cavities were housed in plastic jars under laboratory conditions until  
121 bioassays (see below).

122 *Neoponera villosa* belongs to the neotropical species complex of *N. foetida* (L.), which includes 12  
123 other species [56,57]. Due to their morphological similarity, *N. villosa* has been confused in the past  
124 with two other species with a wide distribution, *N. inversa* (Smith) and *N. curvinodis* (Forel). Until  
125 now, however, only *N. villosa* has been reported in the Yucatan Peninsula [58]. Nevertheless, in order  
126 to confirm ant identity and further support our comparisons, five workers nesting in bromeliads and  
127 five workers nesting in live trees were DNA extracted and barcoded as part of an independent study  
128 (Lachaud and Pérez-Lachaud, unpubl.). DNA extraction and amplification followed the protocol in  
129 [59], with a freezing step after initial incubation according to the recommendation of [60] for  
130 Hymenoptera. Sequences were edited using CodonCode v. 3.0.1 (CodonCode Corporation, Dedham,  
131 MA, USA) and uploaded to Barcode of Life Database (BOLD, boldsystems.org). Voucher specimens  
132 were deposited in the Formicidae Collection of El Colegio de la Frontera Sur at Chetumal, Quintana  
133 Roo, Mexico (ECO-CH-F).

### 134 2.2. Nest site selection

135 As our study is focused on nest relocation, only workers were used for the two-choice tests  
136 implemented to evaluate nest-site selection. This parallels nest relocation following disturbance or  
137 destruction of the old nest whereby emigrations are organized by workers (scouts) that set out from  
138 the damaged nest to find a new home, thoroughly inspecting any candidate nest that they find [2].  
139 As in various other ponerine species such as *N. verenae* (Forel) (referred to as *Pachycondyla*  
140 *obscuricornis* Emery; [61]), *N. apicalis* (Latreille) [62], or *Diacamma indicum* (Santschi) [63,64], *N. villosa*  
141 uses a specific behavior called tandem running in which a single worker attracts a single recruit (or  
142 two in some occasions) and leads her towards the new nest site [17]. As in other *Neoponera* [61,62]  
143 such recruitment by *N. villosa* is exclusively used during nest relocation and never during foraging  
144 [see 18].

## 145 2.2.1. Experimental set up

146 Ants from 35 colonies living in *Ae. bracteata* and 11 colonies (or parts of colonies) nesting in tree  
147 cavities were used in bioassays (Table S1). Observations were conducted from 26 January 2017 to 12  
148 February 2018. Bioassays were carried out two weeks after field collection; during this period of  
149 acclimatization, and during bioassays, ants were held under natural illumination and at room  
150 temperature ( $26 \pm 1$  °C). Workers were randomly selected from the original nest and only evaluated  
151 once. Two-choice tests were conducted using transparent plastic jars (14 cm in diameter x 25 cm in  
152 height, 3 l vol.) into which the ants were deposited. Each jar was connected via a transparent plastic  
153 tube (1.5 cm in diameter x 20 cm in length) to an election chamber (bioassays with live plants: 45 x 30  
154 x 60 cm plastic box; bioassays with parts of plants (leaves): 40 x 21 x 14 cm). Each election chamber  
155 included a glass tube (2 cm in diameter x 8 cm in length) filled with water and stuffed with cotton at  
156 one end. The ants were fed sliced apple pulp, which was placed in the election chamber for the  
157 duration of the bioassay.

158 The protocol for the observations followed that of [25]. Behavioral heterogeneity among colony  
159 members is common in insect societies and individual behavioral specialization during nest moving  
160 has been signaled for example in the model ant genus *Temnothorax* [65,66]; however, specialized  
161 *Temnothorax* workers are readily replaced in removal experiments showing organizational resilience  
162 of ant colonies [65]. As colony size in *N. villosa* varies greatly from one colony to another, and because  
163 it was not feasible to collect a sufficient number of complete colonies to perform the required number  
164 of replicates per bioassay, a fixed sample-size of 20 ants per bioassay was used. This is furthermore a  
165 common procedure in experiments with large ants (see for example: [36,37]). For each bioassay, two  
166 different refuges or “nest-sites”, consisting in tubular shelters to eliminate the influence of plant  
167 architecture, were placed in the election chamber. Subsequently, a group of 20 workers randomly  
168 obtained from those foraging and some brood were gently placed into the adjacent transparent jar.  
169 We carried out 21 to 30 replicates for each comparison and each replicate consisted of individuals  
170 from the same colony. The experimental device was then closed and set aside for 24 h, allowing the  
171 ants to install themselves in one shelter along with the brood (see [36]). The stimulus for the initiation  
172 of movement towards a potential nest (no shelter in the jar and artificial illumination) was constant  
173 across experiments and across replicates within experiments. This is a standard procedure used to  
174 trigger colony relocation in ants [67]. After 24 hours we evaluated the number of workers in any of  
175 the two refuge options (“nest-site”) and those that remained in the jar or that were wandering or  
176 foraging.

## 177 2.2.2. Experiment 1.

178 To evaluate whether *N. villosa* nests in any available cavity or whether it prefers the refuge  
179 provided by *Ae. bracteata*, workers were presented with two tubular shelters (4 cm in diameter x 10  
180 cm in length, with only one opening) made from: a) the rolled leaves of *Ae. bracteata* (treatment) and  
181 b) from a cardboard (control). Thirty replicates were performed with workers originating from  
182 colonies living in bromeliads and thirty with workers from colonies collected in tree cavities.

## 183 2.2.3. Experiment 2.

184 To evaluate whether *N. villosa* workers are able to discriminate *Ae. bracteata* through chemical  
185 stimuli emitted by the plant, shelters made of leaves of two other species belonging also to the  
186 Bromeliaceae family (*Ae. bromeliifolia* (Rudge) Baker, and *Ananas comosus* (L.) Merr.) were offered in  
187 combination with *Ae. bracteata* in two-choice bioassays as in the previous experiment. Thirty  
188 replicates were performed for each comparison with workers originating from colonies living in  
189 bromeliads, and 21 to 26 replicates with ants from colonies collected in tree cavities. For each  
190 replicate, the ants had the choice between two shelters: one shelter made from the leaves of *Ae.*  
191 *bracteata* (control), and another made from the leaves of one of the two other bromeliad species.

## 192 2.2.4. Experiment 3.

193 To evaluate the influence of other bromeliad-related traits (structure of the plant), ants were  
194 offered the choice between whole plants of similar size of the two *Aechmea* species. Thirty replicates  
195 were performed with both types of workers.

#### 196 2.2.5. Experiment 4.

197 As the results of the previous experiments showed that both shelters made of *Ae. bracteata* leaves  
198 and whole plants of this species were preferred (see Results), we evaluated whether the choice by *N.*  
199 *villosa* ants could be influenced by the size of the available *Ae. bracteata*. Twenty-nine replicates with  
200 workers from colonies nesting in *Ae. bracteata* were set up. In each replicate, *Ae. bracteata* bromeliads  
201 of two different sizes were offered: small (25 cm) vs. large (80 cm).

#### 202 2.3. Statistical analyses

203 Not all ants were found inside refuges; some workers were foraging and others were inactive.  
204 Inactivity is very common in social insects and is an intrinsic feature of the ants' behavior [68], making  
205 up to 40% of the members in a colony (e.g. [69]); furthermore, specific workers are consistently  
206 inactive [70]. Inactive workers are quantitatively important in *N. villosa* colonies [19]. To avoid  
207 inconsistencies due to a number of ants not choosing or performing other activities, we calculated the  
208 total number of ants found inside the proposed refuges or plants, and used proportions of ants as the  
209 variable response.

210 The probability of ants being in any of the proposed refuges or plants was analyzed fitting a  
211 Generalized linear mixed model with a binomial error distribution and a logit link (maximum  
212 likelihood). To control for any bias due to colony-level effects, colony was included as a random  
213 effect, and the treatments (with or without experience with *Ae. bracteata*) as a fixed effect. To explore  
214 the magnitude of the fixed effect, we performed a Likelihood ratio test (Wald chi-square test).  
215 Inferences about sample proportions were analyzed with one sample proportion Z tests under the  
216 null hypothesis of random selection ( $p_0 = 0.5$ ). Analyses were performed in package LME4 in R  
217 version 3.6.2 [71, 72].

#### 218 2.4. Ethics statement

219 Sampling comply with the current laws of Mexico and was carried out under permit number  
220 FAUT-0277 from Secretaría de Medio Ambiente y Recursos Naturales, Dirección General de Vida  
221 Silvestre, granted to G.P.-L. Only the biological material required for this study was collected.

### 222 3. Results

#### 223 3.1. Species identification

224 DNA sequences generated in the present work (Genbank accession numbers MK779595 to  
225 MK779604) confirmed that both populations (ants nesting in *Ae. bracteata* and ants nesting in live tree  
226 cavities) did not diverge genetically: all ants used in this work belonged to *N. villosa*. DNA sequences  
227 of both populations represent a single molecular operational taxonomic unit, and cluster with all  
228 other *N. villosa* molecular public data (Figure S1).

#### 229 3.2. Tandem running behavior

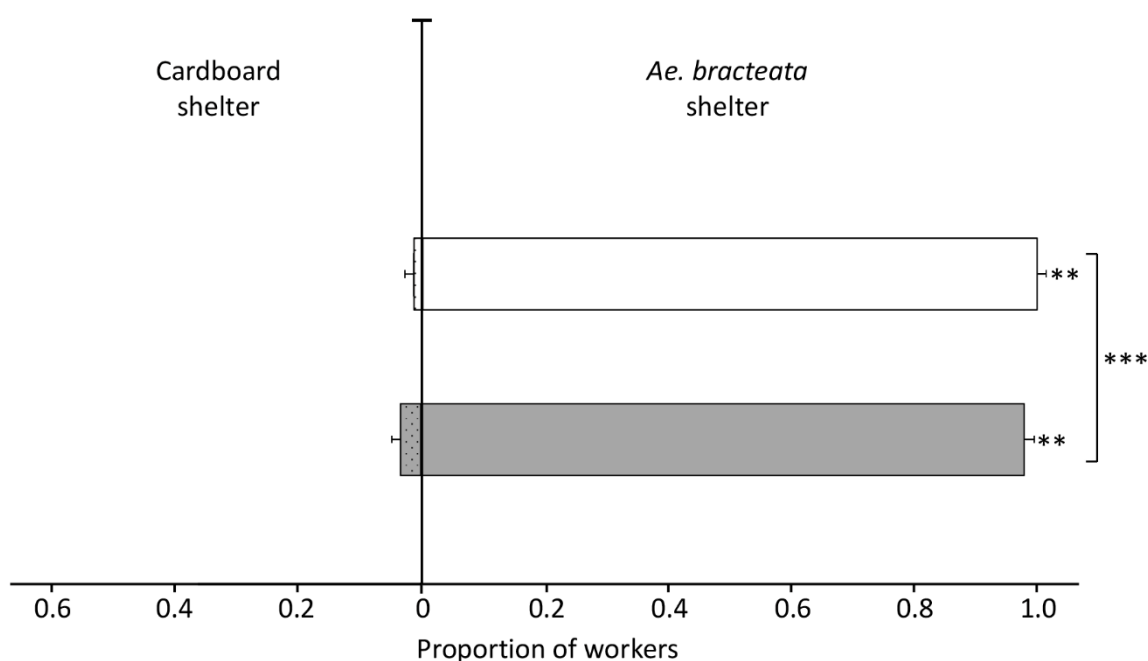
230 In the bioassays, *N. villosa* workers began exploring the new area (election chamber) and the  
231 proposed refuges, then selected one shelter. Afterwards, workers returned to the nest box and  
232 recruited nestmates through tandem running behavior. These recruited workers moved to the  
233 selected refuge, inspected it, and returned to the "old nest" to recruit new nestmates. Qualitatively  
234 we found that several tandem running ants followed the same path, suggesting trail laying behavior,  
235 although marking of the trail was not observed (Video S1). Similar trail laying through hind gut fluids  
236 or pygidial gland secretions has been reported in other ponerine species using tandem running [73–  
237 76]. The recruitment process was initiated through a "jerking" movement of a recruiting ant

238 stimulating a nestmate to follow her to the new nest site, whereby the ant performs a rapid and  
 239 vertical shaking of the body. Such rapid, vertical shaking of the body displayed by the recruiting ant  
 240 to enhance the chemical signal has been reported in other ant species from various subfamilies [77,78].  
 241 Nestmates reacted by replicating the jerking movements and then initiated tandem running along  
 242 the trail of the recruiting ant toward the new nest site. A single worker, or occasionally a maximum  
 243 of two, were recruited and travelled in a single column. In some cases, when contact between the  
 244 scout and the follower was broken, the recruiting ant pulled the legs of the nestmate with their jaws  
 245 to reinforce the recruitment signal.

### 246 3.3. Nest site selection

#### 247 3.3.1. Experiment 1.

248 Ants from both origins (whether originally nesting in bromeliads or in tree cavities) significantly  
 249 preferred refuges made up of the leaves of *Ae. bracteata* over cardboard shelters (one sample Z tests,  
 250  $Z = 5.20$ ,  $p < 0.05$ ; and  $Z = 5.34$ ,  $p < 0.05$  for ants originally nesting in *Ae. bracteata* and those nesting in  
 251 tree cavities, respectively; Figure 1). However, the origin of ants had a significant influence in their  
 252 choices (type II Wald  $\chi^2 = 8338.5$ ,  $df = 1$ ,  $p < 0.0001$ ) as ants originally nesting in bromeliads had a  
 253 significantly lower probability of choosing the refuge made up of leaves of the bromeliad ( $0.978 \pm$   
 254  $0.0002$ , mean  $\pm$  SEM; 95% CI [ $0.9774 - 0.9781$ ]) than ants originally nesting in tree cavities ( $0.989 \pm$   
 255  $0.0001$ ; 95% CI [ $0.9892 - 0.9897$ ]). In general, the brood was transported to the shelters with the higher  
 256 proportion of workers.



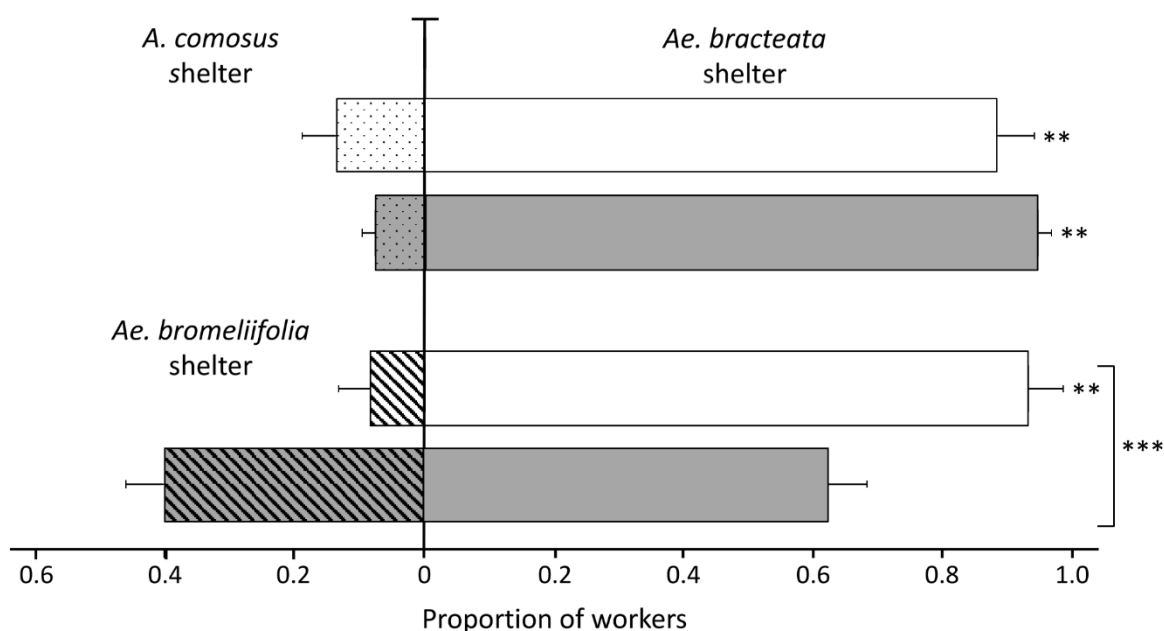
257

258 **Figure 1.** Mean proportion of *N. villosa* workers ( $\pm$  SEM) in shelters consisting of *Ae. bracteata* leaves  
 259 (empty bars) vs. cardboard shelters (dotted bars). Workers from colonies of two distinct origins were  
 260 tested: ants originally nesting in *Ae. bracteata* ( $n = 30$  trials, grey bars), and ants from colonies  
 261 previously nesting in tree cavities ( $n = 30$  trials, white bars). \*\*  $p < 0.05$ , one sample Z test; \*\*\*  $p <$   
 262  $0.0001$ , type II Wald  $\chi^2$ .

#### 263 3.3.2. Experiment 2.

264 When ants had to select between refuges made up of leaves of two *Aechmea* species, their choice  
 265 was significantly influenced by their previous nesting site ( $\chi^2 = 13.067$ ,  $p = 0.00035$ ): ants from tree  
 266 cavities, which did not have any previous contact with *Ae. bracteata* have a higher probability of

267 choosing *Ae. bracteata* over *Ae. bromeliifolia* ( $0.960 \pm 0.027$ , mean  $\pm$  SEM; 95% CI [0.8592 - 0.9895]), than  
 268 ants from colonies originally nesting in *Ae. bracteata* ( $0.581 \pm 0.089$ ; [0.4043 - 0.7391]). Ants from colonies  
 269 nesting in *Ae. bracteata* did not show any marked preference ( $Z = 1.18$ ,  $p > 0.10$ ; Figure 2). However,  
 270 when the choice concerned refuges made up of leaves of species from two different Bromeliaceae  
 271 genera, ants from both origins preferred *Ae. bracteata* over *A. comosus* (ants nesting in bromeliads:  
 272  $0.952 \pm 0.018$ , mean  $\pm$  SEM; 95% CI [0.9015 - 0.9776]; ants in tree cavities:  $0.929 \pm 0.033$ , mean  $\pm$  SEM;  
 273 95% CI [0.8296 - 0.9720]); Figure 2); and although this pattern was stronger in ants originally nesting  
 274 in *Ae. bracteata*, the probability of choosing between *Ae. bracteata* and *A. comosus* was not influenced  
 275 by the origin of ants ( $\chi^2 = 0.486$ ,  $p = 0.48$ ).



276

277 **Figure 2.** Mean proportion of *N. villosa* workers ( $\pm$  SEM) in shelters consisting of leaves of *Aechmea*  
 278 *bracteata* (empty bars) vs. leaves of two other bromeliads: *Ae. bromeliifolia* (stripped bars) or *Ananas*  
 279 *comosus* (dotted bars). Workers from colonies of two distinct origins were tested: workers originally  
 280 nesting in *Ae. bracteata* ( $n = 30$  trials, grey bars), and workers from colonies previously nesting in tree  
 281 cavities ( $n = 21$  or  $n = 26$  trials, white bars). \*\*  $p < 0.05$ , one sample Z test; \*\*\*  $p < 0.0001$ , type II Wald  
 282  $\chi^2$ .

### 283 3.3.3. Experiment 3.

284 The origin of the ants had no effect on the probability of choosing between whole plants of *Ae.*  
 285 *bracteata* and *Ae. bromeliifolia* ( $\chi^2 = 0.011$ ,  $p = 0.916$ ). Ants of both origins preferred *Ae. bracteata* over *Ae.*  
 286 *bromeliifolia* plants (ants originally nesting in *Ae. bracteata*:  $0.661 \pm 0.034$ , mean  $\pm$  SEM; 95% CI [0.5915  
 287 - 0.7249]; ants nesting in tree cavities:  $0.667 \pm 0.041$ , mean  $\pm$  SEM; 95% CI [0.5814 - 0.7430]; Figure 3).



288

289 **Figure 3.** Mean proportion of *N. villosa* workers ( $\pm$  SEM) in bioassays of whole plants of *Ae. bracteata*  
 290 (empty bars) vs. *Ae. bromeliifolia* (dotted bars) of the same size. Bioassays were performed with two  
 291 different experimental groups: workers originating from colonies originally nesting in *Ae. bracteata* (n  
 292 = 30 trials, grey bars) and workers from colonies collected in tree cavities (n = 30 trials, white bars). \*  
 293  $p < 0.05$ ; \*\*\*  $p < 0.01$ ; one sample Z test.

294 3.3.4. Experiment 4.

295 *Neoponera villosa* workers originating from colonies established in *Ae. bracteata* significantly chose  
 296 large *Ae. bracteata* bromeliads over small ones (One sample Z test,  $Z = 4.30$ ,  $p < 0.01$ ; Figure 4). The  
 297 mean proportion of ants choosing the large over the small bromeliad was 0.899; 95% CI [0.8489 -  
 298 0.9510]



299



300 **Figure 4.** Mean proportion of *N. villosa* workers ( $\pm$  SEM) in bioassays with two *Ae. bracteata* of different  
301 sizes: large (80 cm) vs. small (25 cm). All workers tested were originally nesting in *Ae. bracteata* (n =  
302 29 trials). One sample Z test, \*\*  $p < 0.01$ .

#### 303 4. Discussion

304 Most animals, if not all, exhibit innate behaviors in response to specific sensory stimuli [79,80].  
305 Bumble bees and honeybees, for example, exhibit innate color preferences, notably yellow and  
306 purple, which reflects the peak sensitivity of their color receptors [81–84], while the  
307 pseudomyrmecine ant *Tetraoponera aethiops* Smith shows an innate attraction to its natural host plant  
308 *Barteria fistulosa* Mast (Passifloraceae) even when young callows were reared in laboratory conditions  
309 without any further contact with this plant post emergence [37]. Our experiments provide a similar  
310 example of innate attraction of *N. villosa* workers towards the bromeliad *Ae. bracteata*. Whatever the  
311 origin of the ants (with or without previous experience with *Ae. bracteata*), our data show that *N.*  
312 *villosa* workers exhibit a significant preference for refuges made up of leaves of *Ae. bracteata* over other  
313 available potential refuges consisting of leaves of another bromeliad species, *A. comosus*, or of  
314 cardboard, but ants with a previous experience with *Ae. bracteata* did not differentiate between  
315 refuges made up of leaves of this bromeliad and those of the close *Ae. bromeliifolia*. Contrary to the  
316 results obtained by [25], which did not demonstrate any spontaneous preference of *N. villosa* for *Ae.*  
317 *bracteata* (workers reared in the laboratory without any contact with plants were attracted  
318 indifferently towards *Ae. bracteata* or towards the orchid *Myrmecophila tibicinis* (Batem.) Rolfe), our  
319 results point to the existence of an innate preference for *Ae. bracteata*. In all of the bioassays, workers  
320 without previous experience with this bromeliad significantly preferred *Ae. bracteata*, although a  
321 proportion of ants did not engage in emigrations (inactive ants).

322 The influence of the environment, through pre-imaginal and neonatal learning (early  
323 experience), can interfere and replace any innate attraction or repulsion [85,86]. In ants,  
324 environmental induction of adult choices by passive familiarization during early adulthood has been  
325 demonstrated for various species. For example, although under natural conditions thyme (*Thymus*  
326 *vulgaris* L.) repels adult workers of the formicines *Formica polyctena* Foerster and *Camponotus vagus*  
327 Scopoli, workers of these species chose to settle in tubes that contained this plant if they have been  
328 reared in its presence when they were callow neonates [35,87]. Similarly, in the African arboreal ants  
329 *T. aculeatum* and *O. longinoda*, early learning during the first part of the life of adult workers and  
330 gynes can supersede innate attraction to guava (*Psidium guajava* L.) and cocoa (*Theobroma cacao* L.) or  
331 to mango leaves (*Mangifera indica* L.), respectively [36]. Early learning during the larval life and just  
332 after adult emergence appears to occur to some extent in *N. villosa*, as our data show that the  
333 preference for *Ae. bracteata* was modulated by the original nesting substrate: previous experience with  
334 *Ae. bracteata* enhancing the preference towards this plant over cardboard or *A. comosus*. These results  
335 confirm previous studies on foundresses of *N. villosa* which have shown that the influence of the  
336 original nest site environment on subsequent individual choice during nest site selection for colony  
337 foundation is due to an imprinting during larval life, strengthened at the beginning of the imago life  
338 through early learning [25]. When given the choice between a refuge containing *Ae. bracteata* or  
339 nothing, gynes of *N. villosa* that have previously experienced contact with the bromeliad during their  
340 larval and the first part of their nymphal life significantly preferred *Ae. bracteata*, whereas gynes  
341 which completed their nymphal life on another epiphyte (*M. tibicinis*) did not discriminate between  
342 both options. However, gynes which completed their nymphal life on *M. tibicinis* displayed a  
343 significant preference to this plant when presented with a choice between *Ae. bracteata* and *M. tibicinis*  
344 [25]. The local fidelity towards *Ae. bracteata* over other available nest-sites observed in our study area  
345 and the marked preference for this epiphyte during our experiments might be explained through  
346 early learning by winged queens as suggested by [25], combined to an innate attraction to *Ae. bracteata*  
347 in workers, influencing their choice during nest relocation events. The evolution of such an innate  
348 attraction, leading to local specialization in *Ae. bracteata* as a nesting-site, may be due to the  
349 predominance of this particular bromeliad throughout the biogeographic area of *N. villosa* (*Ae.*  
350 *bracteata* occurs from E Mexico to N Colombia and NW Venezuela [88]), and also because this

351 bromeliad as microhabitat provides specific benefits to ants, both as shelter and as foraging site, and  
352 further constitutes refuge during extreme stochastic climatic events [33,89]. Preference in *N. villosa*  
353 workers for *Ae. bracteata* may be an adaptive response driven by climatic events in our study area,  
354 promoting the selection of such a stable microhabitat.

355 Various other bromeliad associated traits (size, chemical profile) may also be involved in nest-  
356 site selection as demonstrated by the preference of *N. villosa* workers for large *Ae. bracteata* when  
357 presented with small and large plants, or by the differences in preference demonstrated when  
358 choosing between closely related bromeliad species (both when offered as refuges and complete  
359 plants), most likely related to the chemical substances they produce. The bromeliads used in this  
360 study share similar traits (e.g. long and narrow leaves), and the texture of their leaves and general  
361 architecture are similar, but not identical; furthermore, the nature and composition of their chemical  
362 signals are different, particularly between species from different genera [90], and it is possible that  
363 ants use any small difference in structure or composition of the leaves during nest-site selection. In  
364 addition, the architectural form and complexity of the plant may facilitate the emergence of different  
365 temperature and humidity microhabitats suitable for ants to settle, and it is known that other  
366 specialist arthropods (e.g. bromeliad-dwelling salticids) choose bromeliads based on rosette and leaf  
367 architectures [91]; furthermore, larger individual plants with a complex structure may facilitate the  
368 development of large ant populations and promote the maintenance of a diverse community of  
369 potential prey [92]. The use of chemical cues for host localization has been reported in a wide range  
370 of insects, including both herbivores [93–96] and predators or parasites [97–99]. In ants, chemical  
371 volatiles are also used to identify potential host plants. For example, *Crematogaster* spp. foundress  
372 queens can recognize their *Macaranga* host plant species, identifying chemical compounds of the stem  
373 surfaces of seedlings [46]; and queens of *Azteca* spp. and *Allomerus octoarticulatus* Mayr use chemical  
374 cues to select their myrmecophyte *Cordia nodosa* Lam. [47]. In our experiments, it was evident that *N.*  
375 *villosa* workers originally nesting in *Ae. bracteata*, had difficulty in arriving at a consensus when in the  
376 presence of shelters of two *Aechmea* species, without further information on the suitability of the  
377 potential nest-sites (plant structure or size of the cavity, for example) and therefore, the probabilities  
378 of choosing either bromeliad were similar (Fig. 2). Contrastingly, choosing between complete plants  
379 of these two bromeliads was straight forward and significantly in favor of *Ae. bracteata* over the other  
380 species, although some ants also settled inside *Ae. bromeliifolia* plants. Noteworthy, a proportion of  
381 ants did not engage in emigrations (inactive ants). Our failure to show any statistical difference when  
382 ants chose between refuges of these two bromeliads might have arisen, in part, due to the presence  
383 of inactive workers and the possible exclusion of specialized workers in our reduced experimental  
384 groups. This may have influenced the number of ants in shelters after 24h, as a significant higher  
385 latency in performing emigration tasks has been reported for workers not specialized on emigration  
386 tasks in *Temnothorax* ants [65]. Considered together, these results suggest that workers of *N. villosa*  
387 select *Ae. bracteata* through some plant stimuli, probably of chemical nature, supplemented with  
388 information provided by the whole plant. The ability to discriminate between distinct plant species  
389 and genera is an obvious advantage, as the time and energy to find a suitable nest-site is minimized.

390 Evidently, nearly all ant species have the capability of shifting their nests if they become  
391 unsuitable [1], and selecting the best nest-site among numerous alternatives may be critical to the  
392 success and survival of the colony. As our results show, in most instances *N. villosa* workers preferred  
393 to settle in *Ae. bracteata* shelters and plants over other possibilities, and preferred large *Aechmea* plants  
394 over small ones. Behavioral flexibility constitutes an essential component of the adaptive repertoires  
395 of animals. In this context, it is not impossible that modulation of the innate attraction of *N. villosa*  
396 workers towards *Ae. bracteata* through early experience may facilitate the replacement of this plant as  
397 the most suitable nest-site in habitats where *Ae. bracteata* is rare, as occurs in the northern part of the  
398 Yucatan Peninsula where this species nests in cavities of several live trees.

## 399 5. Conclusions

400 In our experiments, nest-site choice by *N. villosa* workers was influenced by an innate attraction  
401 to the bromeliad *Aechmea bracteata*. The local fidelity towards *Ae. bracteata* over other available nest-

402 sites in the southern region of the Yucatan Peninsula and the marked preference for this epiphyte in  
 403 our experiments might be explained through this innate attraction to *Ae. bracteata* influencing the  
 404 choice of workers during nest relocation events. Reinforcement of this preference by pre-imaginal  
 405 and early learning during adulthood is not excluded, but more experiments are needed. Preference  
 406 of *N. villosa* workers for *Ae. bracteata* may be an adaptive response driven by extreme climatic events  
 407 in our study area, promoting the selection of such a stable microhabitat as nesting-site.

408 **Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1): Table S1. Original  
 409 composition of the *Neoponera villosa* colonies used in the two-choice bioassays. Figure S1. Taxon ID tree of *N.*  
 410 *villosa* molecular public data, including the 10 sequences from this study (highlighted in yellow). Video S1.  
 411 Characteristic recruitment behavior in *Neoponera villosa*. Note that two tandem pairs are following the same path,  
 412 suggesting the existence of chemical trail laying.

413 **Author Contributions:** Conceptualization, Jean-Paul Lachaud and Gabriela Pérez-Lachaud; Data curation,  
 414 Franklin H. Rocha and Gabriela Pérez-Lachaud; Formal analysis, Franklin H. Rocha and Gabriela Pérez-  
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 419 Franklin H. Rocha and Gabriela Pérez-Lachaud; Writing – review & editing, Jean-Paul Lachaud, Yann Hénaut,  
 420 Carmen Pozo and Gabriela Pérez-Lachaud. All authors have read and agreed to the published version of the  
 421 manuscript.

422 **Funding:** This work was partially supported by the Consejo Nacional de Ciencia y Tecnología, Mexico  
 423 (scholarship grant 269825 to F.H.R.).

424 **Acknowledgments:** We are grateful to Javier Valle-Mora, César Raziél Lucio-Palacios, and Alma Estrella García-  
 425 Morales (ECOSUR) for statistical advice, help with ant collection, and DNA extraction and amplification,  
 426 respectively. This work was partially supported by the Consejo Nacional de Ciencia y Tecnología, Mexico  
 427 (scholarship grant 269825 to FHR). We acknowledge the facilities provided by Sian Ka'an Biosphere Reserve to  
 428 collect ants (Biol. Angel Omar Ortiz Moreno).

429 **Conflicts of Interest:** The authors declare no conflict of interest. The funders had no role in the design of the  
 430 study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to  
 431 publish the results.

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