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Pablo Servigne, Jérôme Orivel, Frédéric Azémar, James Carpenter, Alain Dejean, et al.. An uneasy alliance: a nesting association between aggressive ants and equally fierce social wasps. *Insect Science*, 2020, 27 (1), pp.122-132. 10.1111/1744-7917.12597 . hal-02322753

HAL Id: hal-02322753

<https://hal.science/hal-02322753>

Submitted on 7 Oct 2021

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1 An uneasy alliance: a nesting association between aggressive ants and 2 equally fierce social wasps

3
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6
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13
14 **Short running title:** An ant-wasp nesting association

15
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18
19 **Abstract** Although the Neotropical territorially dominant arboreal ant *Azteca chartifex* Forel is
20 very aggressive towards any intruder, its populous colonies tolerate the close presence of the fierce
21 polistine wasp *Polybia rejecta* (F.). In French Guiana, 83.33% of the 48 *P. rejecta* nests recorded
22 were found side by side with those of *A. chartifex*. This nesting association results in mutual
23 protection from predators (i.e., the wasps protected from army ants; the ants protected from birds).
24 We conducted field studies, laboratory-based behavioral experiments and chemical analyses to
25 elucidate the mechanisms allowing the persistence of this association. Due to differences in the
26 cuticular profiles of the two species, we eliminated the possibility of chemical mimicry. Also,
27 analyses of the carton nests did not reveal traces of marking on the envelopes. Because ant forager
28 flows were not perturbed by extracts from the wasps' Dufour's and venom glands, we rejected any
29 hypothetical action of repulsive chemicals. Nevertheless, we noted that the wasps 'scraped' the
30 surface of the upper part of their nest envelope using their mandibles, likely removing the ants'
31 scent trails, and an experiment showed that ant foragers were perturbed by the removal of their
32 scent trails. This leads us to use the term 'erasure hypothesis'. Thus, this nesting association
33 persists thanks to a relative tolerance by the ants towards wasp presence and the behavior of the
34 wasps that allows them to 'contain' their associated ants through the elimination of their scent
35 trails, direct attacks, 'wing-buzzing' behavior and ejecting the ants.

36
37 **Key words** *Azteca chartifex* ants; interspecific association; mutualism; *Polybia rejecta* wasps;
38 scent trail erasure

39 40 41 Introduction

42
43 Predation by ants, particularly army ants (Ecitoninae), is known to have a major impact on
44 the ecology and evolution of Neotropical social wasps (Richards & Richards, 1951; Jeanne, 1975,
45 1979, 1991; Young, 1979; Chadab-Crepet & Rettenmeyer, 1982). Wasps have noticeably evolved
46 different means of defense against predatory ants such as (1) the selection of a safe nest site and
47 using long thorns to anchor their nest (Jeanne, 1975; Dejean *et al.*, 1998a), (2) the architectural
48 properties of their nests which may be protected by an envelope or connected to the substrate
49 through a long, thin, easy-to-defend petiole (Jeanne, 1975), and (3) adaptive behavior such as
50 constant vigilance, instantaneous escape, rapid movements, repellent chemicals smeared onto the
51 nest petiole, the active guarding of the nest entrance, and agonistic defense with the use of venom
52 or by ejecting the ants (Jeanne, 1970, 1975, 1991; Post & Jeanne, 1981; Kojima, 1993; Dejean *et*
53 *al.*, 1998b; Coutinho Togni & Giannotti, 2010; Grangier & Lester, 2011).

54 However, many social wasp species protect themselves from army ants by, paradoxically,
 55 living in close proximity to certain species of arboreal ants, which implies appropriate nest site
 56 selection by foundresses or swarms. Indeed, by constantly patrolling their host tree, arboreal ants
 57 ‘control’ their branches and leaves and may be able to exclude other ants, including army ants
 58 (Chadab-Crepet & Rettenmeyer, 1982; Herre *et al.*, 1986; Dejean *et al.*, 1998b; Corbara *et al.*,
 59 2009). For instance, workers of the territorially dominant arboreal ant *Azteca chartifex* defend
 60 access to their host tree by attacking army ants at its base, causing the columns to deviate (Chadab-
 61 Crepet & Rettenmeyer, 1982).

62 Although they probably concern a limited number of wasp species, examples of associations
 63 with ants are easily found in the Neotropics. For instance, the social wasps *Synoeca surinama*, *S.*
 64 *virginea*, *Chartergus chartarius*, *Polistes pacificus*, *Agelaia myrmecophila* and *Polybia rejecta*
 65 nest close to *Azteca* ants (Richards, 1978; Jeanne, 1991; Corbara *et al.*, 2009; Souza *et al.*, 2013;
 66 Somavilla *et al.* 2013). Also, *Protopolybia emortualis* nests in close vicinity to nests of the ant
 67 *Dolichoderus bidens* (Corbara *et al.*, 2009, 2018), whereas some other social wasp species nest on
 68 plants called myrmecophytes that shelter the colonies of a limited number of ant species in hollow
 69 structures (Herre *et al.*, 1986; Corbara *et al.*, 2009; Dejean *et al.*, 2012). Until now, little has been
 70 known about the mechanisms allowing the wasps to live in close contact with arboreal ants which,
 71 as notable predators, may represent a constant threat to the wasps and their brood.

72 Because they are commonly associated with arboreal ants, mostly *A. chartifex*, the nests of
 73 the polistine wasp *P. rejecta* are protected from army ant raids (Richards, 1978; Chadab-Crepet &
 74 Rettenmeyer, 1982; Corbara *et al.*, 2009; Souza *et al.*, 2013). In turn, the wasps protect the ant
 75 nests from bird predation (Le Guen *et al.*, 2015). Because *A. chartifex* are territorially dominant
 76 arboreal ants, Jeanne (1991) suggested that ‘*these wasps need to keep a constant vigil to keep*
 77 *Azteca out of their nests, so the alliance seems to be an uneasy one*’.

78 The present study aims to understand the mechanisms underlying this ‘uneasy alliance’ by
 79 assessing three alternative scenarios. The ‘chemical mimicry hypothesis’ is based on the fact that
 80 social insects discriminate colony mates from aliens through cuticular hydrocarbon (CHC)
 81 signatures. Some invertebrates are adapted to living in association with ants based on a cuticular
 82 profile matching that of their associated ants through biosynthesis (chemical mimicry) or
 83 acquisition (chemical camouflage) (Lenoir *et al.*, 2001). The congruence of the CHC signatures of
 84 ants and associated social wasps permitting their reciprocal tolerance was noted by Espelie and
 85 Hermann (1988). In the two other scenarios (which are not mutually exclusive), ants and wasps do
 86 not tolerate interspecific contact; the cohabitation may thus be based on exclusion mechanisms
 87 initiated by the wasps. In the ‘repulsive chemical markings hypothesis’, the wasps spread repellent
 88 chemicals on the wasp nest surface (see Jeanne, 1970, 1991) and in the ‘ant-trail erasure
 89 hypothesis’, the wasps remove the ants’ scent trails (see West-Eberhard, 1989). In order to test
 90 these hypotheses, we conducted a field survey complemented by laboratory-based behavioral
 91 experiments and the chemical analyses of ant and wasp cuticular compounds and carton nests.

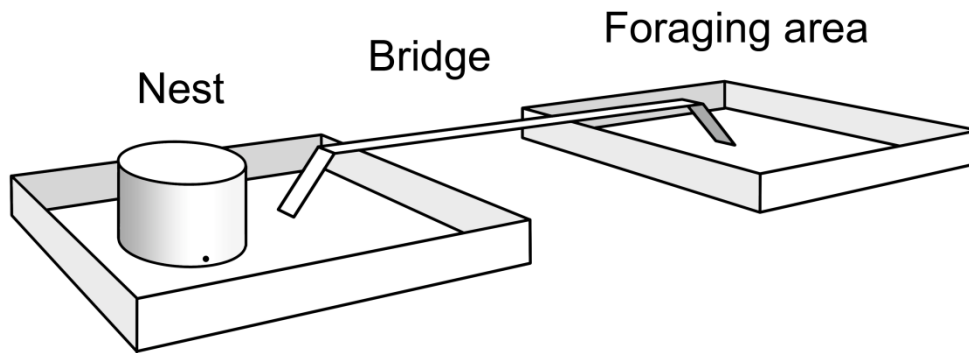
92 **Materials and methods**

93 *Species*

94 Polydomous colonies of the territorially dominant arboreal ant *A. chartifex* Forel (Dolichoderinae)
 95 occur in clusters of several large (up to 2 m in height) pendant carton nests that can shelter
 96 millions of individuals. They are common in primary forest as well as in secondary areas,
 97 especially along corridors such as roads. Workers (2- to 3-mm long) are very aggressive and
 98 defend their territories day and night (Delabie *et al.*, 1991; Longino, 2007).
 99

100 The swarm-founding social wasp *P. rejecta* (F.) (Vespididae, Polistinae, Epiponini)
 101 (individuals \approx 15mm in length) is common in Amazonian rainforests. It has populous colonies of
 102 several hundred individuals living in nests protected by an envelope and reaching 2 m in height,
 103 although most of them measure less than 70 cm; they can form aggregates of up to 23 wasp nests
 104 (Jeanne, 1978, 1991; Corbara *et al.*, 2009; Dejean *et al.* 2017b). Workers, which hunt soft-bodied
 105

106 arthropods, are very aggressive, and their sting is extremely painful for humans (Richards, 1978;
107 Starr, 1985; Dejean *et al.*, 2017b).



108
109 **Fig. 1** Experimental set-up. The bridge is 30-cm long and 1-cm in width.
110

111 *Sampling and study area*

112 The climate of the area studied is moist tropical with 3400 mm of yearly precipitation typically
113 recorded at Petit-Saut, French Guiana (5°03'39"N, 53°02'36"W). The maximum and minimum
114 monthly temperatures average 33.5°C and 20.5°C, respectively. The dry season occurs between
115 July and November, and the rainy season between December and June with a marked decrease in
116 precipitation during March.

117 During a field campaign conducted from March 2003 to March 2015, we inspected ≈12 km
118 of forest edges in the districts of Sinnamary and Kourou in French Guiana (altitude ranging from
119 10 to 100 m a.s.l.). We thoroughly inspected each plant taller than 0.75 m and noted each time we
120 found a *P. rejecta* nest its width and length, the distance from the ground and if it was associated
121 with ants. If so, we noted the species of ant and the distance between the *P. rejecta* nest and the
122 closest nest of the associated ant. We especially noted the *Azteca chartifex* colonies because *P.*
123 *rejecta* is mostly associated with this ant species.
124

125 **Table 1** Characteristics of the *Polybia rejecta* nests including their size, distance from the ground (A) and
126 relationships with ants (B), with special reference to their relationships with *Azteca chartifex* colonies (C).
127

(A) <i>Polybia rejecta</i> nest characteristics								
Width	No. cases	%	Length	No. cases	%	Distance from the ground	No. cases	%
15-20 cm	35	72.92	15-30 cm	21	43.75	< 1.5 m	4	8.33
20-30 cm	10	20.83	30-50 cm	16	33.33	1.5-4 m	12	25.00
> 30 cm	3	6.25	50-70 cm	7	14.58	4-10 m	23	47.92
			> 70 cm	4	8.33	10-20 m	9	18.75
Total	48	100	Total	48	99.99	Total	48	100

(B) Associations with ants			
Associated ant species	No. cases	%	Special reference to <i>Azteca chartifex</i> colonies
Without associated ants	6	12.50	No. <i>Azteca chartifex</i> colonies recorded: 68
With <i>Dolichoderus bidens</i>	1	2.08	
With <i>Dolichoderus bispinosus</i>	1	2.08	No. <i>A. chartifex</i> colonies associated with <i>P. rejecta</i> : 29 (42.65%)
With <i>Azteca chartifex</i>	40	83.33	
Total	48	99.99	

(C) Details on the relationships between <i>Polybia rejecta</i> and <i>Azteca chartifex</i> nests						
No. of <i>P. rejecta</i> nests per <i>A. chartifex</i> colonies	No. cases	No. wasp nests	%	Distance from the <i>A. chartifex</i> nest	No. cases	%
				Contact	3	7.5
1 nest	22	22	55.0	< 10 cm	14	35.0
2 nests	5	10	25.0	10-20 cm	14	35.0
3 nests	1	3	7.5	20-30 cm	7	17.5
5 nests	1	5	12.5	> 30 cm	2	5.0
Total	29	40	100	Total	40	100

128 Voucher specimens of *P. rejecta* were deposited in the American Museum of Natural
129 History (New York), and *A. chartifex* were deposited in the *Laboratório de Mirmecologia*
130 (CEPEC-CEPLAC, Itabuna, Bahia, Brazil).

131

132 *Ethograms*

133 In the field, two *P. rejecta* nests constructed near large *A. chartifex* nests were the subject of
134 behavioral observations. We used an anti-wasp suit to approach these nests. After the wasps
135 became used to our presence, we placed, 0.5 m from the nests, a black cloth screen (1.5 x 2 m)
136 with a small plastic window. After two days the wasps were not aggressive if we remained behind
137 the screen, allowing us to observe their behavior without wearing the suit (which is extremely hot
138 in the tropics).

139 Preliminary observations permitted us to establish a list of behavioral items (Table 1). Then,
140 eight 20-min-long periods of observation carried out between 9:40 and 17:00 (the final 20 minutes
141 of each hour), allowed us to establish ethograms with transition frequencies. Ethograms were
142 drawn for a 'normal' situation (no disturbance) and a 'defensive' situation after we disturbed the
143 *A. chartifex* ants by slightly tapping on the twig supporting their nests with a stick. Consequently,
144 numerous *A. chartifex* ants left their nests, invading the neighboring branches, including those
145 supporting the wasp nests, so that some of them stepped on the upper part of the wasp nest
146 envelope (hereafter called the 'anchoring zone') triggering a response from the wasps.

147

148 *Gas chromatographic analyses of workers and nest cartons*

149

150 Nine *P. rejecta* x *A. chartifex* associations were analyzed. For each of them, five wasps and 20
151 ants were freeze-killed and their abdomens were removed in order to avoid chemical
152 contamination by the Dufour's and venom glands.

153 Moreover, their carton nests were analyzed in order to detect any possible chemical
154 compounds produced by the wasps to deter ants. If such repellent compounds do exist, the wasps
155 probably spread them along the top of the nest, which is in close proximity to the supporting
156 branch where the ants are likely to walk. For each association, we collected two 1 x 1 cm samples
157 of carton (1) from the bottom of the wasp nest envelope and (2) from the 'anchoring zone' of the
158 wasp nest.

159 Worker and carton samples were immersed in 1 ml of hexane for 5 min (hexane is used as a
160 solvent to extract the CHCs). The extracts were then evaporated under nitrogen and dissolved in
161 200 µl of hexane for the *P. rejecta* workers, 100 µl for the *A. chartifex* workers and 50 µl for the
162 pieces of carton nest. A sample of 2 µl of these solutions was analyzed with a Hewlett-Packard
163 5890 Series II gas chromatograph equipped with a split-splitless injector, a flame ionization
164 detector, and a nonpolar fused-silica capillary column (HT-5, 25m x 0.22 mm ID x 0.1 µl film
165 thickness). Sample injections were performed in splitless mode using helium as the carrier gas,
166 with injector and detector temperatures at 300°C and 320°C, respectively. The oven temperature
167 program was as follows: 100°C to 320°C at a rate of 5°C/min, and then isothermal (320°C) for 10
168 min. Integrations were performed with Millennium 2.15 software (Waters).

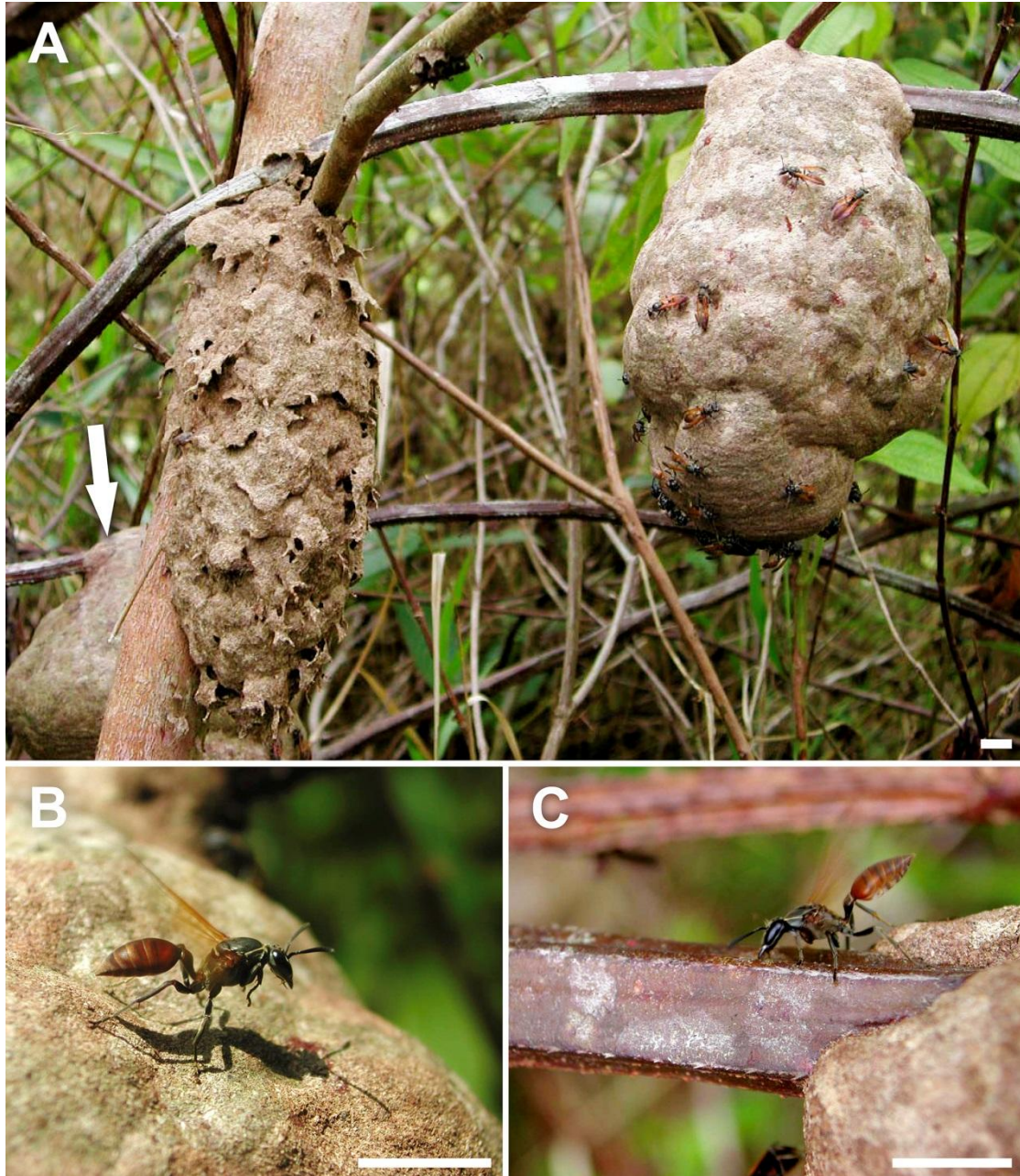
169 A Factorial Correspondence Analysis was conducted based on the cuticular profiles of *A.*
170 *chartifex* and *P. rejecta* workers and the carton of their nests (including the 'anchoring zone' and
171 the lower part of the envelope for *P. rejecta* nests). To estimate the similarities of the profiles, the
172 relative percentage of each peak was calculated. A hierarchical cluster analysis using Ward's
173 method for aggregation (i.e., analysis of variance to evaluate the distances; Ward, 1963) was then
174 conducted. Similarities between groups were also estimated by calculating their Nei's distances
175 (Nei, 1972; SPAD 3.01 software).

176

177 *Ant-flow disruption in the field*

178 This survey concerned a wasp nest which hung from a horizontal branch (B) 30 cm from the
179 associated ant nest. In order to find out whether the presence of the wasp nest modified the

180 itineraries of the foraging ants or not, we measured their flows (i.e., number of workers per 5 min)
 181 on branch B which the ants used to walk over the wasp nest ‘anchoring zone’ and on another
 182 horizontal branch (A) serving as a control. Both branches started from the ant nest and led to
 183 neighboring shrubs where the ants attended hemipterans. The statistical comparison was conducted
 184 using the Mann-Whitney test (GraphpadPrism 5.0 software).
 185



186
 187

188 **Fig. 2** (A) A *Polybia rejecta* nest covered with its brownish envelope in close vicinity to an *Azteca*
 189 *chartifex* nest made of light gray carton. Note the presence of a second wasp nest almost in contact with the
 190 ant nest (see arrow). (B) ‘Wing-buzzing’ behavior. (C) ‘Scraping’ behavior on the branch supporting the
 191 wasp nest after *A. chartifex* workers walked on it. The white scales represent 1 cm.
 192

193 *Ant-flow disruption in the laboratory*

194 Because the wasps might disturb the ants by depositing a repellent compound or by permanently
 195 scraping off the ants’ scent trails, we conducted the following experiments using an *A. chartifex*
 196 nest previously associated with wasps in the field. This nest was harvested and then installed in a
 197 terrarium whose inner walls were coated with Fluon® to prevent the ants from climbing out. This
 198 terrarium was linked to a foraging arena containing diluted honey by a plastic bridge (10 mm in

199 width; 30 cm in length) interconnecting the center of the terrarium and the center of the foraging
200 arena) (see Fig. 1). Within a few minutes, there was a column of workers crossing the bridge.

201 A first series of experiments consisted in placing in the middle of the bridge a 1 x 1 cm piece
202 of paper. We compared a control lot (the pieces of paper were only dipped in acetone and then
203 dried; two replicates; ten 2.5-minute-long observations) with two experimental lots. To prepare the
204 latter, we dissected wasps from two colonies to isolate their Dufour's and venom glands (including
205 the venom sac in the latter case). We tested the action of the compounds from the wasps' Dufour's
206 gland (each time, the contents of three Dufour's glands were diluted in acetone and then dried; 10
207 replicates). Then, the same procedure was conducted using the contents of three venom glands.
208 We compared the ant flows under these three conditions during the 30 seconds following the
209 introduction of the paper strips using an ANOVA (Shapiro-Wilk normality tests passed;
210 GraphpadPrism 5.0 software).

211 A second series of experiments consisted in testing the reaction of the ants when confronted
212 with the absence of a scent trail. We placed in the middle of the bridge 10-cm-long, 1-cm-wide
213 paper strips and let the ants mark them during \approx 30 minutes (i.e., until the flow stabilized) and we
214 measured the ant flow during 30 seconds. Then, we replaced these marked strips by virgin strips of
215 the same size and again measured the ant flow for 30 seconds. As the ants marked these new
216 strips, we again measured their flow 10 minutes later (for 30 seconds). This process was repeated
217 30 times during 10 non-consecutive days. We compared the results using a Repeated Measures
218 ANOVA followed by a Tukey *post-hoc* test (Shapiro-Wilk normality tests passed; GraphpadPrism
219 5.0 software).

220
221 **Table 2** Behavioral acts performed by ants and wasps in ethograms from Fig. 3.
222

Ants: <i>Azteca chartifex</i>	Wasps: <i>Polybia rejecta</i>
- Turns back: the ant stops on the branch before the wasp nest and turns back without touching it.	- Wing-buzzing: (Fig. 2b) faced with an ant, the wasp stands up and creates a whirlwind with its wings held forward which moves the ants antennae and makes them turn back (considered a defense behavior; Jeanne 1991). When the ants are numerous, wasps ventilate in a group (<i>group fanning behavior</i>).
- Goes across: the ant climbs onto the wasp nest, goes across and continues on the branch.	- Ignoring: contact does not induce any behavior.
- Goes down: the ant climbs onto the wasp nest and walks down toward the wasp nest entrance, always situated at the bottom of the envelope.	- Pecking: the wasp hits the ant with its mandibles.
- Escapes: after contact, the ant changes its direction and flees from the wasps.	- Ejections: When an ant bites a wasp, the latter takes the ants between its mandibles, flies up and drops the ant. This behavior has also been observed in <i>Polybia occidentalis</i> (Jeanne 1991), <i>Parachartergus apicalis</i> (Dejean <i>et al.</i> 1998) and <i>Vespula vulgaris</i> (Grangier and Lester 2011).
- Ignores: contact does not induce any behavior.	
- Bites: the ant bites a wasp leg.	- Scraping: (Fig. 2c) the wasps scrape the carton nest with their mandibles and antennate its surface.

223

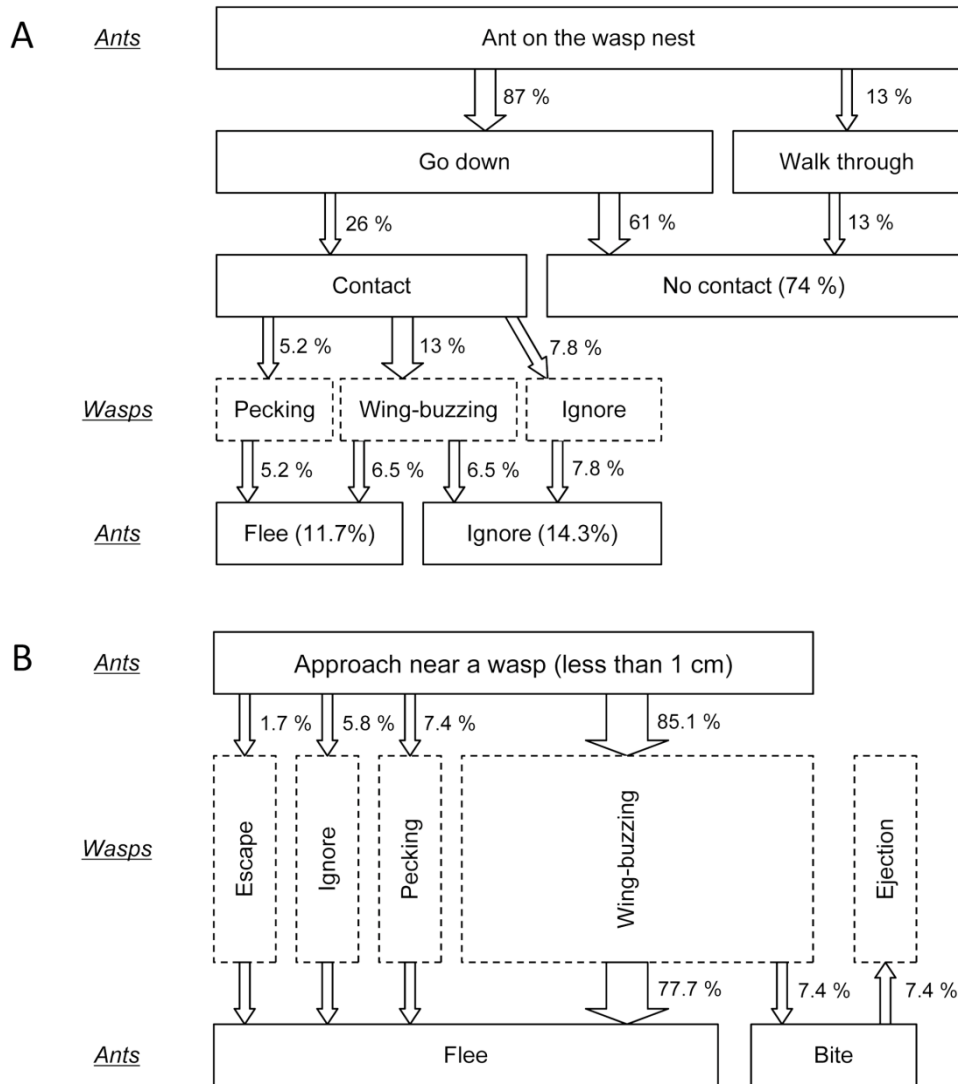
224 Results

225

226 *Specificity of the association*

227 During the field survey we recorded 48 *P. rejecta* nests (details of their characteristics and their
228 associations with ants are provided in Table 1); six of them were not associated with ants
229 (12.50%), one was associated with *Dolichoderus bidens* (2.08%), another with *Dolichoderus*
230 *bispinosus* (2.08%) and the 40 remaining (83.3 %) with *A. chartifex* (Table 1; Fig. 2). In the latter

231 case, seven ant colonies were associated with two to five *P. rejecta* nests; the distance between the
 232 ant and the wasp nests, less than 10 cm in 42.5% of the cases, can reach 70 cm (Table 1).
 233



234
235

236 **Fig. 3** Ethogram representing the behavioral sequences of an encounter between *Azteca chartifex* ants and
 237 *Polybia rejecta* wasps. (A) In 'normal' conditions on the upper part of the wasp nest envelope (N = 154
 238 cases). (B) After exciting the *A. chartifex* ants by tapping a stick on their nest (N = 121). Percentages were
 239 calculated based on the overall number of cases; 4 h of observation in each situation.

240 The gas chromatographic analyses of cuticular and carton compounds showed three distinct
 241 categories: ant workers, wasp workers and carton (Fig. 4A). Qualitative comparisons of the
 242 profiles using Nei's distances confirmed these results (Table 2).
 243

243

244 Ethograms

245 We sometimes observed ants walking on the upper part of the wasp nest envelope. When
 246 approaching the wasp nest, the ants intermittently stopped, their gaster raised, and antennated the
 247 upper part of the wasp nest envelope. Among the ants starting to walk on the branch supporting the
 248 wasp nest, 130 out of 284 (45.8%) went back to their nest (no contact with wasp individuals),
 249 whereas the 154 others (54.2%) walked over the wasp nest 'anchoring zone', 40 of them coming
 250 into contact with the wasps. The wasp reacted by wing-buzzing (20 cases), ignoring (12 cases) or
 251 pecking (8 cases), and the ants ignored them (22 cases) or fled (18 cases) (see the ethogram in Fig.
 252 3A). None of these ants recruited nestmates.

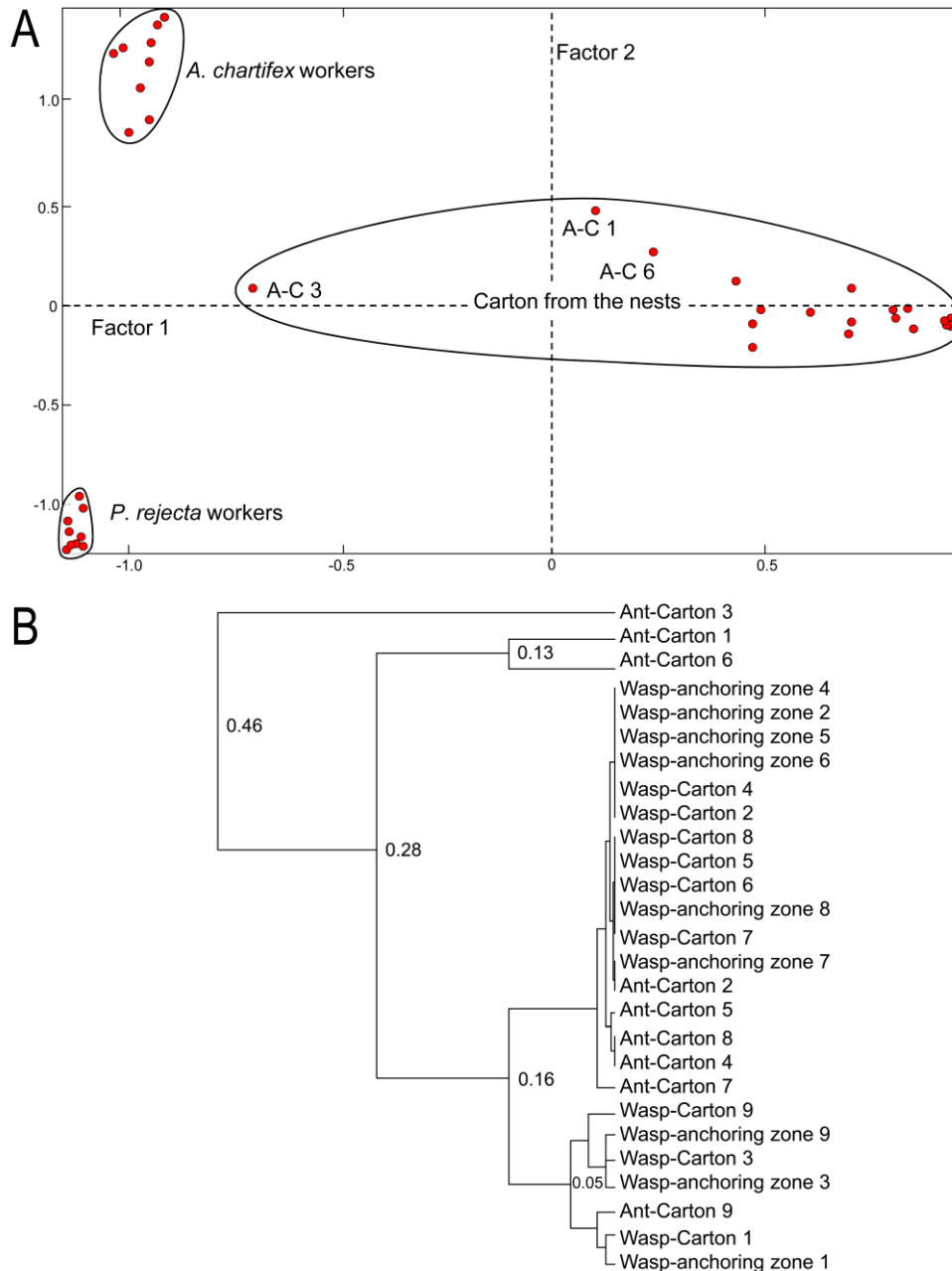
253

254 When the ants were artificially disturbed, hundreds of workers left their nest, invaded all the
 neighboring branches, and climbed onto the wasp nest, so that ant-wasp contact was frequent. In

255 reaction, numerous recruited wasps left their nest displaying aggressive behavior such as pecking,
 256 wing-buzzing (see Fig. 2B) and ejecting the ant in 112 out of the 121 encounters noted (92.5%)
 257 (see the ethogram in Fig. 3B). During the period of ant excitation and the following 5 to 10
 258 minutes, several wasps scraped the ‘anchoring zone’ of the nest (see “scraping” behavior in Fig.
 259 2C).

260 Carton groups analyzed with a hierarchical cluster analysis showed no difference between
 261 the profiles of the carton from the ‘anchoring zone’ and from the lower part of the wasp nest
 262 envelope (Fig. 3B). We also noted a resemblance between the profiles of the ant carton and the
 263 wasp carton from both the ‘anchoring zone’ and the rest of the envelope for five associations (2, 4,
 264 5, 7, 8), and strong differences for the three other associations (1, 3, 6) (Fig. 4B).

265



266 **Fig. 4** (A) Factorial correspondence analysis of the cuticular profiles of *Azteca chartifex* and *Polybia*
 267 *rejecta* workers as well as the carton of their nests (for *P. rejecta* nests, the ‘anchoring zone’ and the lower
 268 part of the envelope are represented). Axes 1 and 2 explain 29.97% and 21.30% of the variance,
 269 respectively (A-C 1, A-C 3, A-C 6: ant carton from nests N° 1, 3 and 6, see below). (B) Hierarchical cluster
 270 analysis of the cuticular profiles of the carton of *A. chartifex* and *P. rejecta* (lower part and upper part or
 271 ‘anchoring zone’ of the wasp nest envelope). Node values are expressed in terms of dissimilarity whose
 272 index varies from 0 (identical profiles) to 1.
 273

274 **Table 3** Average Nei's distances (mean \pm SE) as a measure of similarity in the cuticular profiles
 275 between *Azteca chartifex* and *Polybia rejecta*. Nei's distances were calculated from binary values
 276 (presence/absence) in each recorded peak and vary between 0 (totally different) and 1 (identical)
 277 (* carton gathered from the 'anchoring zone').
 278

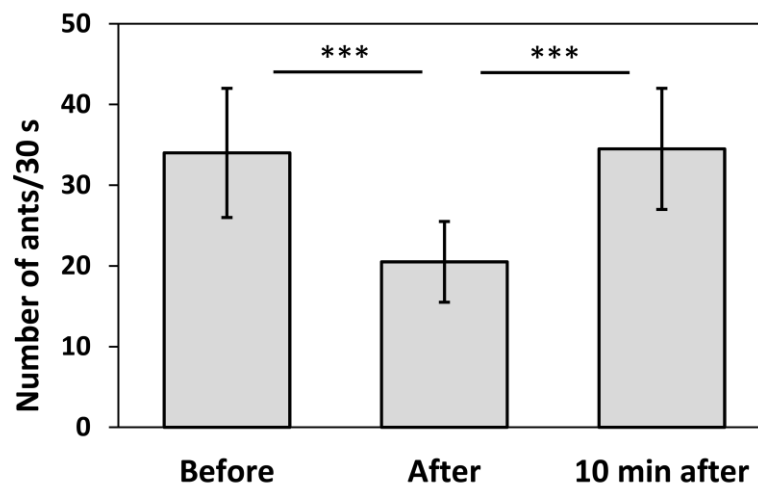
	<i>A. chartifex</i> workers	<i>A. chartifex</i> carton	<i>P. rejecta</i> workers	<i>P. rejecta</i> lower carton	<i>P. rejecta</i> upper carton
<i>A. chartifex</i> workers	0.98 \pm 0.01	0.57 \pm 0.02	0.49 \pm 0.01	0.48 \pm 0.02	0.48 \pm 0.02
<i>A. chartifex</i> carton	—	0.94 \pm 0.02	0.50 \pm 0.01	0.75 \pm 0.02	0.76 \pm 0.03
<i>P. rejecta</i> workers	—	—	0.99 \pm 0.01	0.54 \pm 0.03	0.55 \pm 0.02
<i>P. rejecta</i> lower carton	—	—	—	0.95 \pm 0.02	0.94 \pm 0.02
<i>P. rejecta</i> upper carton*	—	—	—	—	0.96 \pm 0.02

279

280 *Ant-flow disruption*

281 In the field, the presence of a wasp nest disrupts the ant flow as we noted 126.7 \pm 21.1 ants / 5 min
 282 on branch A (without a wasp nest; mean \pm SD), whereas no ant walked on branch B in 20 cases out
 283 of 25; the highest value being 9 ants / 5 min (n=25 in both cases; Mann-Whitney test: Z = 6.2; P<
 284 0.0001). From the very low rate of workers using branch B, we deduced that the wasps can deter
 285 the ants, but this might be by depositing a repellent compound or by permanently scraping off their
 286 scent trails.

287 During the first laboratory experiment, the ants were not 'stopped' by the wasps' Dufour's
 288 and venom gland extracts and walked over the strips of paper very much as they did for the control
 289 (strip of paper dipped in acetone and then dried), so that the range of flow varied from 19 to 38
 290 ants per 30 seconds (N = 10 in each case; mean \pm SD: control: 28.4 \pm 2.8 ants per 30 seconds;
 291 Dufour's gland extract: 26.0 \pm 7.04 ants per 30 seconds; venom gland extracts: 26.0 \pm 3.5 ants per 30
 292 seconds; statistical comparisons ANOVA: F_{2,29} = 0.82; P = 0.45; NS).
 293



294

295 **Fig. 5** Ant flow (number of ants in 30 sec; means \pm SD) before and after the replacement of a marked paper
 296 strip with a virgin paper strip and 10 minutes later. Statistical comparisons; repeated measures ANOVA: F_{2,}
 297 ₈₉ = 39.48; P<0.0001; Tukey's *post-hoc* test: A vs. B, P<0.001; A vs. C, N.S.; B vs. C, P<0.001).
 298

299

300 After the 10-cm-long strips of paper marked by the ants were replaced with virgin strips,
 301 most ants stopped and raised their gaster and some of them returned to their nest, so that the ant
 302 flow decreased significantly (Fig. 5). Yet, after 10 minutes, when the ants had marked this new
 303 strip of paper, their flow returned to a value similar to that of the control (Fig. 5).

304 Discussion

305
306 In this study we provide new information on the characteristics of *P. rejecta* nests and confirm that
307 they are commonly associated with *A. chartifex* colonies (concerns 83.33 % of *P. rejecta* nests;
308 N=48), but this mutualistic association is not obligatory for either partner (see also Jeanne, 1978;
309 Richards, 1978; Corbara *et al.*, 2009). The distance between *A. chartifex* and *P. rejecta* nests
310 varied between less than 10 cm and 70 cm, a larger range than previously reported (i.e., 10-30 cm;
311 Souza *et al.*, 2013; Somavilla *et al.*, 2013; Virgínio *et al.*, 2015).

312 In this ‘uneasy alliance’, which the wasps initiate by installing their nests in the vicinity of
313 an *A. chartifex* carton nest, wasp nest architecture plays an important role in the defense against
314 ants, particularly the envelope combined with a small entrance that is easy to defend (Jeanne,
315 1991). Nevertheless, wasp nest protection relies on active behavioral defenses coupled with the
316 recruitment of nestmates onto the nest envelope elicited by chemicals from the venom gland
317 (Jeanne, 1975, 1991, 1996; London & Jeanne, 1996). For *P. rejecta*, these defenses can be direct
318 attack, ‘wing-buzzing’ and ejecting the ants as noted for some other social wasp species (see also
319 Post & Jeanne, 1981; Jeanne, 1991; Dejean *et al.*, 1998b; London & Jeanne, 2005; Coutinho Togni
320 & Giannotti, 2010; Grangier & Lester, 2011), especially if the *A. chartifex* ants are excited by
321 external disturbance.

322 In undisturbed conditions, our experiments also showed a very low rate of ants patrolling the
323 branch passing through the wasp nest ‘anchoring zone’ compared to the activity of ants on nearby
324 branches, suggesting that the wasps have evolved an indirect way of repelling the ants. Also, *A.*
325 *chartifex* ants generally fled when, while walking on the *P. rejecta* nest ‘anchoring zone’, they
326 encountered wasp individuals. Indeed, in undisturbed conditions these ants are never aggressive
327 toward the wasps and they never recruited nestmates although they are territorially dominant and
328 able to deter army ant raids (see Chadab-Crepet & Rettenmeyer, 1982).

329 *The chemical mimicry hypothesis*

330
331 We noted that the relationship between *P. rejecta* wasps and *A. chartifex* workers is based on
332 agonistic reactions from the wasps, something consistent with the different cuticular profiles of
333 these ants and wasps (see Fig. 4A). We can therefore reject the chemical mimicry hypothesis that
334 requires cuticular profile congruence as is known for the *Parachartergus apicalis* x
335 *Pseudomyrmex* associations (Espelie & Hermann, 1988). Other cases of chemical mimicry are
336 well known between ants and myrmecophiles (Akino, 2008; Vantaux *et al.*, 2010; Dejean *et al.*,
337 2017a).

338
339 Concerning the carton of the ant and wasp nests, the influence of the fibers constituting them
340 is likely primordial (see the importance of the host plant compounds in an ant-wasp association in
341 Espelie & Hermann, 1988). Indeed, we never noted differences between the envelope and the
342 ‘anchoring zone’ of the wasp nests (likely due to the use of fibers from the same tree for each
343 wasp nest) (Fig. 3B). The carton of the ant nests can be similar to both (and not only the
344 ‘anchoring zone’, the only part that can be marked by the *A. chartifex* ants) or completely different
345 (Fig. 3B), making it difficult to detect through gas chromatography (particularly if the carton was
346 scraped by the wasps). Thus, the similarities or differences between ant and wasp carton likely
347 originate in the fibers gathered to build the nests (i.e., from similar or different plants,
348 respectively) rather than compounds originating from the individuals involved in the nest
349 construction (i.e., saliva and other compounds that are specific).

350 *The repulsive chemical markings hypothesis*

351
352 A repellent ant barrier on the nest petiole is a defense mechanism found in independent-founding
353 polistines (produced from a gland at the base of the sixth metasomal sternite) and some
354 Stenogastrinae (from the Dufour’s gland) (Jeanne, 1996; London & Jeanne, 2000). In contrast,
355 swarm-founding wasps do not use such a chemical barrier except for *Nectarinella championi* and

356 *Leipomeles dorsata* that deposit sticky droplets around their nest entrance or the supporting leaf
 357 petiole access, respectively (Jeanne, 1991; Wenzel, 1991).

358 Our data suggest that *P. rejecta* do not use repulsive markings to deter ant presence. Indeed,
 359 chromatographic analyses revealed no difference between compounds on the upper and the lower
 360 parts of a wasp nest. Moreover, the Dufour's and venom glands of *P. rejecta* have no effect on the
 361 *A. chartifex* flow. Interestingly, we observed three times that a few hours after a *P. rejecta* colony
 362 absconded, the *A. chartifex* workers invaded the abandoned wasp nests, excluding the possibility
 363 of persistent repellent markings. Thus, we have no argument in favor of the repellent markings
 364 hypothesis.

365 *The ant-trail erasure hypothesis*

366 In the present study, three facts support the ant-trail erasure hypothesis formulated by West-
 367 Eberhard (1989) who showed that *Metapolybia aztecoides* wasps scraped the carton of their nests
 368 to remove *Azteca* sp. scent trails. First, some *A. chartifex* scouts came regularly onto the wasp nest
 369 'anchoring zone' switching from trail-walking to a searching behavior. Second, *P. rejecta* wasps
 370 very often scraped the top of their nest, particularly just after an ant walked on the surface of the
 371 wasp nest or when an ant alarm was artificially triggered, as is known for *Clypearia sulcata*, *M.*
 372 *aztecoides* and *P. occidentalis* (Chadab-Crepet & Rettenmeyer, 1982; West-Eberhard, 1989;
 373 Jeanne, 1991). Third, in laboratory conditions, the ants reacted strongly to the 'erasure' of the
 374 pheromone trail (i.e., U-turns, raising the gaster) suggesting that *A. chartifex* workers are very
 375 sensitive to the absence of their scent trails.

376 Even if the existence of this mechanism is not (or cannot be) definitively proved, it provides
 377 the most compelling explanation concerning the constantly very low level of ant presence on *P.*
 378 *rejecta* nests. This is in accordance with the fact that the gas chromatographic analyses showed no
 379 difference between the profiles of the carton from the 'anchoring zone' and from the lower part of
 380 the wasp nest envelope (Fig. 4B), countering the hypothesis that the wasps deposit a secretion
 381 while scraping. Nevertheless, future studies dealing with the erasure process of ant trails should
 382 test the role of the wasps' exocrine glands whose secretions are likely involved in chemical
 383 defense (see Fortunato *et al.*, 2000 for the mandibular glands).

384 In conclusion, the *P. rejecta* x *A. chartifex* nesting association, which is initiated when the
 385 wasps install their nests, requires an important level of tolerance by the associated ants, but they
 386 can be aggressive when disturbed by an external source. This association persists thanks to this
 387 relative tolerance maintained by the wasp's constant containment behavior.

389 **Acknowledgements**

390 We are grateful to the *Laboratoire Environnement de Petit Saut* (HYDRECO) for logistical help,
 391 Jacques H.C Delabie for the identification of the ants and Andrea Yockey-Dejean for her
 392 participation in the field studies and proof-reading an early version of manuscript. Financial
 393 support for this study was provided by an "*Investissement d'Avenir*" grant managed by the French
 394 *Agence Nationale de la Recherche* (CEBA, ref. ANR-10-LABX-25-01).

396 **Disclosure**

397 All authors declare no conflict of interests.

399 **References**

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