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

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

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

Introduction

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

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

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

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

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

Materials and methods

Species

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

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

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

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

Sampling and study area

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

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

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

(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.</i>			
With <i>Azteca chartifex</i>			40	83.33	<i>rejecta</i> : 29 (42.65%)			
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	

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

Ethograms

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

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

Gas chromatographic analyses of workers and nest cartons

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

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

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

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

Ant-flow disruption in the field

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

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

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

Ant-flow disruption in the laboratory

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

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

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

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

Table 2 Behavioral acts performed by ants and wasps in ethograms from Fig. 3.

Ants: <i>Azteca chartifex</i>	Wasps: <i>Polybia rejecta</i>
<ul style="list-style-type: none"> - Turns back: the ant stops on the branch before the wasp nest and turns back without touching it. - Goes across: the ant climbs onto the wasp nest, goes across and continues on the branch. - 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. - Escapes: after contact, the ant changes its direction and flees from the wasps. - Ignores: contact does not induce any behavior. - Bites: the ant bites a wasp leg. 	<ul style="list-style-type: none"> - 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>). - Ignoring: contact does not induce any behavior. - Pecking: the wasp hits the ant with its mandibles. - 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). - Scraping: (Fig. 2c) the wasps scrape the carton nest with their mandibles and antennate its surface.

Results

Specificity of the association

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

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

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

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

Ethograms

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

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

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

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

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

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

	<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

Ant-flow disruption

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

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

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

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

Discussion

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

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

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

The chemical mimicry hypothesis

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

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

The repulsive chemical markings hypothesis

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

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

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

The ant-trail erasure hypothesis

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

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

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

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Disclosure

All authors declare no conflict of interests.

References

- Akino, T. (2008) Chemical strategies to deal with ants: a review of mimicry, camouflage, propaganda, and phytomimesis by ants (Hymenoptera: Formicidae) and other arthropods. *Myrmecological News*, 11, 173–181.
- Chadab-Crepet, R. and Rettenmeyer, C.N. (1982) Comparative behavior of social wasps when attacked by army ants or other predators and parasites. *The biology of social insects* (eds. M.D. Breed, C.D. Michener & H.E. Evans), pp 155-171. Westview Press, Boulder, CO.

- Corbara, B., Carpenter, J.M., Céréghino, R., Leponce, M., Gibernau, M. and Dejean, A. (2009) Diversity and nest site selection of social wasps along Guianese forest edges: assessing the influence of arboreal ants. *Comptes Rendus Biologies*, 332, 470–479.
- Corbara, B., Servigne, P., Dejean, A., Carpenter, J.M. and Orivel J. (2018) A mimetic nesting association between a timid social wasp and an aggressive arboreal ant. *Comptes Rendus Biologies*, doi:10.1016/j.crv.2018.01.006.
- Coutinho Togni, O. and Giannotti, E. (2010) Colony defense behavior of the primitively eusocial wasp, *Mischocyttarus cerberus* is related to age. *Journal of Insect Science*, 10, 136.
- Dejean, A., Azémar, F., Libert, M., Compin, A., Hérault, B., Orivel, J., Bouyer, T. and Corbara, B. (2017a) Ant-lepidopteran associations along African forest edges. *The Science of Nature*, 104, 7.
- Dejean, A., Carpenter, J.M., Corbara, B., Wright, P., Roux, O. and LaPierre L.M. (2012) The hunter becomes the hunted: when cleptobiotic insects are captured by their target ants. *Naturwissenschaften*, 99, 265–273.
- Dejean, A., Corbara, B. and Carpenter, J.M. (1998a) Nesting site selection by wasps in the Guianese rain forest. *Insectes Sociaux*, 45, 33–41.
- Dejean, A., Corbara, B. and Lachaud J.P. (1998b) The anti-predator strategies of *Parachartergus apicalis* (Vespidae: Polistinae). *Sociobiology*, 32, 477–487.
- Dejean, A., Rodríguez-Pérez, H., Carpenter, J.M., Azémar, F. and Corbara B. (2017b) The predatory behavior of the Neotropical social wasp *Polybia rejecta*. *Behavioural processes*, 140, 161–168.
- Delabie, J.H.C., Benton, F.P. and De Medeiros, M.A. (1991) La polydomie chez les Formicidae arboricoles dans les cacaoyères du Brésil : optimisation de l'occupation de l'espace ou stratégie défensive ? *Actes des Colloques Insectes Sociaux*, 7, 173–178.
- Espelie, K.E. and Hermann, H.R. (1988) Congruent cuticular hydrocarbons: biochemical convergence of a social wasp, an ant and a host plant. *Biochemical Systematics and Ecology*, 16, 505–508.
- Fortunato, A., Turillazzi, S. and Delfino, G. (2000) Ectal mandibular gland in *Polistes dominulus* (Christ) (Hymenoptera, Vespidae): ultrastructural modifications over the secretory cycle. *Journal of Morphology*, 244, 45–55.
- Grangier, J. and Lester, P.J. (2011) A novel interference behaviour: invasive wasps remove ants from resources and drop them from a height. *Biology Letters*, 7, 664–667.
- Herre, E.A., Windsor, D.M. and Foster, R.B. (1986) Nesting associations of wasps and ants on lowland Peruvian ant-plants. *Psyche*, 93, 321–330.
- Jeanne, R.L. (1970) Chemical defense of brood by a social wasp. *Science*, 168, 465–466.
- Jeanne, R.L. (1975) The adaptiveness of social wasp nest architecture. *Quarterly Review of Biology*, 50, 267–287.
- Jeanne, R.L. (1978) Intraspecific nesting associations in the Neotropical social wasp *Polybia rejecta*. *Biotropica*, 10, 234–235.
- Jeanne, R.L. (1979) A latitudinal gradient in rates of ant predation. *Ecology*, 60, 1211–1224.
- Jeanne, R.L. (1991). The swarm-founding Polistinae. *The social biology of wasps* (eds. K.G. Koss & R.W. Matthews), pp. 389–425. Cornell Univ. Press, Ithaca, NY.
- Jeanne, R.L. (1996) Evolution of exocrine gland function. *Natural History and evolution of paper-wasps* (eds. S. Turillazzi & M.J. West-Eberhard), pp. 144–160. Oxford University Press, Oxford, UK.
- Kojima, J. (1993) A latitudinal gradient in intensity of applying ant-repellent substance to the nest petiole in paper wasps (Hymenoptera: Vespidae). *Insectes Sociaux*, 40, 403–421.
- Le Guen, R., Corbara, B., Rossi, V., Azémar, F. and Dejean A. (2015) Reciprocal protection from natural enemies in an ant-wasp association. *Comptes Rendus Biologies*, 338, 255–259.
- Lenoir A., D'Ettorre P., Errard C. and Hefetz A. (2001) Chemical ecology and social parasitism in ants. *Annual Review of Entomology*, 46, 573–599
- London, K.B. and Jeanne, R.L. (1996) Alarm in a wasp-wasp nesting association: do members signal cross-specifically? *Insectes Sociaux*, 43, 211–215.
- London, K.B. and Jeanne, R.L. (2000) The interaction between mode of colony founding, nest architecture and ant defense in polistine wasps. *Ethology Ecology and Evolution*, 12, 13–25.
- London, K.B. and Jeanne, R.L. (2005) Wasps learn to recognize the odor of local ants. *Journal of the Kansas Entomological Society*, 78, 134–141.
- Longino, J.T. (2007) A taxonomic review of the genus *Azteca* in Costa Rica and a global revision of the *aurita* group. *Zootaxa*, 1491, 1–63.
- Nei, N. (1972) Genetic distances between populations. *American Naturalist*, 106, 283–292.

- Post, D.C. and Jeanne, R.L. (1981) Colony defense against ants by *Polistes fuscatus* in Wisconsin. *Journal of the Kansas Entomological Society*, 54, 599–615.
- Richards, O.W. (1978) *The social wasps of the Americas excluding the Vespinae*. British Museum (Natural History).
- Somavilla, A., Fernandes, I.O., de Oliveira, M.L. and Silveira, O.T. (2013) Association among wasps' colonies, ants and birds in Central Amazonia. *Biota Neotropica*, 13, 308–313.
- Souza, M.M., Pires, E.P. and Prezoto, F. (2013) Nidification of *Polybia rejecta* (Hymenoptera: Vespidae) associated to *Azteca chartifex* (Hymenoptera: Formicidae) in a fragment of Atlantic Forest, in the state of Minas Gerais, southeastern Brazil. *Biota Neotropica*, 13, 390–392.
- Starr, C.K. (1985) A simple pain scale for field comparison of Hymenopterans stings. *Journal of Entomological Science*, 20, 225–232.
- Vantaux, A., Roux, O., Magro, A., Tene Ghomsi, N., Gordon, R.D., Dejean, A. and Orivel J. (2010) Host-specific myrmecophily and myrmecophagy in the tropical coccinellid *Diomus thoracicus* in French Guiana. *Biotropica*, 42, 622–629.
- Virgínio, F., Maciel, T.T. and Barbosa, B.C. (2015) Nidificação de *Polybia rejecta* (Fabricius) (Hymenoptera: Vespidae) associada à *Azteca chartifex* Forel (Hymenoptera: Formicidae) em ecótono de bioma Caatinga/Mata Atlântica, no estado do Rio Grande do Norte. *EntomoBrasilis*, 8, 242–245.
- Ward, J.H. (1963) Hierarchical grouping to optimize an objective function. *Journal of the American Statistical Association*, 58, 236–244.
- Wenzel, J.W. (1991) Evolution of nest architecture. *The social biology of wasps* (eds. K.G. Koss & R.W. Matthews), pp. 480–519. Cornell Univ. Press, Ithaca, NY.
- West-Eberhard, M.J. (1989) Scent-trail diversion, a novel defense against ants by tropical social wasps. *Biotropica*, 21, 280–281.
- Young, A.M. (1979) Attacks by the army ant *Eciton burchelli* on nests of the social paper wasp *Polistes erythrocephalus* in northeastern Costa Rica. *Journal of the Kansas Entomological Society*, 52, 759–768.