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Crematogaster abstinens and *Crematogaster pygmaea* (Hymenoptera: Formicidae: Myrmicinae): from monogyny and monodomy to polygyny and polydomy

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Abstract

Polygyny and polydomy are key features in the nesting biology of many ants, raising important questions in social insect biology, in particular about the ecological determinants of such derived traits. One relevant way to investigate those questions is the comparative study of closely related species with contrasting colony structure. In our study, we investigated and compared morphological (morphology of queens, workers and males), chemical (cuticular hydrocarbon profiles), behavioral (attractiveness of queens and substrates chemically marked by queens, colony foundation, mating behavior), and colony-structure (nest architecture, queen number, nest number) traits in *Crematogaster abstinens* FOREL, 1899 and *C. pygmaea* FOREL, 1904, two related Neotropical species. Our aim was to provide evidence of close evolutionary relationship between the two species and to give new insight into the ecological significance of the polygynous and polydomous system found in *C. pygmaea*. We first showed that the two species share important traits supporting a close evolutionary relationship: same basic morphology in workers and queens, with high queen / worker dimorphism, and ground-dwelling habit with almost identical nest architecture. The two species also share a significant part of their cuticular hydrocarbon (CHC) profiles, which possibly explains the observed attractiveness of substrates marked by queens of one species to workers of the other species. However, the two species completely differ in colony structure: highly polygynous and polydomous colonies in *C. pygmaea;* small, monogynous and monodomous colonies in *C. abstinens*. They also show, in addition to the shared compounds, significant differences in their CHC profiles and differ in mating and colony foundation behavior. In previous studies, the probable existence of a dual dispersal strategy in *C. pygmaea* was shown: a long-range dispersal strategy followed by independent colony foundation and a short-range dispersal strategy through budding events associated with seasonal polygyny and polydomy. We hypothesize that in *C. pygmaea*, polygyny and polydomy could represent a combined evolutionary response to efficiently explore and rapidly saturate patchily distributed habitats that are unstable and subject to a strong seasonality. We also speculate that the polygynous and polydomous colony structure observed in *C. pygmaea* is a derived condition from the monogyny and monodomy observed in *C. abstinens*.

Key words: Integrative taxonomy, cryptic species, ultrastructure, colony structure, dispersal and reproductive strategies, ants.

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Introduction

Queen and nest number in mature colonies are key features of ant colony structure (STEINER & al. 2009). Monogyny (one single queen per colony) and monodomy (the whole colony is housed in a single nest) are thought to be the ancestral states (HÖLLDOBLER & WILSON 1977, ROSS & CARPENTER 1991, KELLER & VARGO 1993, DEBOUT & al. 2007, BOULAY & al. 2014). Monogyny, associated with monandry (queens singly mated) and haplodiploidy, is also thought to be one of the key factors that favored the initial evolution of kin selection, reproductive altruism and, in fine, eusociality in ants (HÖLLDOBLER & WILSON 1990, STRASSMANN 1991, HUGHES & al. 2008).

However, although monogyny and monodomy remain the predominant traits in most ant species (HÖLLDOBLER & WILSON 1977, 1990, KELLER & VARGO 1993, DEBOUT & al. 2007), polygyny (more than one reproductively ac-

tive queen per colony) and polydomy (the colony occupies two or more spatially separated but socially connected nests) are widespread derived traits that seem to have independently and repeatedly evolved in almost all ant subfamilies (HÖLLDOBLER & WILSON 1977, 1990, ROSS & CARPENTER 1991, KELLER & VARGO 1993, DEBOUT & al. 2007, HEINZE 2008, HEINZE & FOITZIK 2009, BOULAY & al. 2014).

The ecological determinants of polygyny and polydomy, and the consequences of such derived traits on social and genetic structure of ant colonies are the subjects of many studies and reviews, especially with reference to polygyny (HÖLLDOBLER & WILSON 1977, 1990, HERBERS 1993, KELLER 1993, 1995, HEINZE & FOITZIK 2009, DEBOUT & al. 2007, ROBINSON 2014). One relevant way to investigate such issues is the comparative study of closely related species with contrasted colony structure (monogyny / polygyny, monodomy / polydomy) (HÖLLDOBLER & WILSON 1977, TSUJI & TSUJI 1996, DEBOUT & al. 2007).

Crematogaster pygmaea FOREL, 1904 and *C. abstinens* FOREL, 1899, two closely related Neotropical species (LONGINO 2003, QUINET & al. 2009), could be promising candidates for such a comparative study. Both species belong to the *Orthocrema* clade (± 140 species) (sensu BLAIMER, 2012a, b), a strongly divergent group in the *Crematogaster* LUND, 1831 genus (467 species), and formed by the former members of subgenera *Orthocrema* SANTSCHI, 1918 (except *C. irritabilis* F. SMITH, 1860 and *C. polita* F. SMITH, 1865), *Neocrema* SANTSCHI, 1918, *Eucrema* SANTSCHI, 1918, *Rhachiocrema* MANN, 1919, and, in part, *Mesocrema* SANTSCHI, 1928) (BLAIMER 2012a, b). The two species are morphologically so closely related that *C. pygmaea* has been synonymized under *C. abstinens* (see LONGINO 2003), before being revived from synonymy, based on the analysis of new collections and on new data on *C. pygmaea* natural history (QUINET & al. 2009).

Crematogaster pygmaea is a highly polygynous and polydomous ground-dwelling ant that is a habitat specialist in coastal and tabuleiro zones of the states of Ceará (QUINET & al. 2009) and Piauí (R. Feitosa, unpubl.) (northeastern Brazil), the only places where this species has been found so far. Usually found in open, urban (or semi-urban) areas with sparse herbaceous vegetation, colonies of *C. pygmaea* are formed by tens of underground nests, each with several queens (mean of 4 queens / nest, up to 36 queens / nest), and interconnected by surface trails used by workers to move from one nest to the other and to reach herbaceous plants where they collect nectar from floral or extrafloral nectaries, and/or honeydew from hemipteran colonies (QUINET & al. 2009, HAMIDI & al. 2012, CARLOS 2015). Nests are relatively simple, at least during the rainy season, formed by a single straight vertical tunnel ± 30 cm in length, with one to four horizontal chambers (QUINET & al. 2009). *Crematogaster pygmaea* polydomous networks have a seasonal component, with colonies undergoing a strong reduction in the number of nests and queens during the dry season, and swiftly expanding during the rainy season, when the number of queens and nests increases (QUINET & al. 2009, CARLOS 2015).

Paradoxically, other features of *Crematogaster pygmaea* biology are more typically found in monogynous species, like strong queen / worker dimorphism (QUINET & al. 2009, PEETERS & al. 2013) and ability of young queens to found colonies in independent and claustral conditions, with strong weight loss in queens during the colony founding stage (HAMIDI & al. 2017). Others are occurrence of nuptial flights (HAMIDI & al. 2017), presence of colony boundaries (multicoloniality) (HAMIDI & al. 2012), and high attractiveness of queens to workers (MARTINS SEGUNDO & al. 2012). Such apparent paradox seems to derive from a dual dispersal strategy in *C. pygmaea*, including a long-range dispersal strategy (mating flights) followed by independent colony foundation (foundation strategy), and a short-range dispersal strategy through budding events that follow fecundation of female sexuals in the parental colony, resulting in expansion of polydomy in colonies (foraging strategy) (HAMIDI & al. 2017).

Crematogaster abstinens has a much wider range, with registered presence in Panama, Colombia, Venezuela, Guyana, French Guiana, Brazil, Bolivia, and Argentina, mainly in dry forest habitats (LONGINO 2003). In the littoral zone of the state of Ceará (northeastern Brazil), it can be found living sympatrically with *C. pygmaea* (QUINET & al. 2009). However, basic aspects of *C. abstinens* biology, including nesting habit (arboreal or ground-dwelling species), colony structure (nest architecture, queen and nest number in colonies), and queen and male morphology, remained unknown (LONGINO 2003).

One of the main difficulties in species delimitation in ants is the large number of hyperdiverse taxa with discrete morphological variation, as observed in *Crematogaster* (BLAIMER 2012b). In fact, some ant genera contain between 40% and 70% of the so-called "cryptic" species, whose status prevents significant advances concerning their biology and ecological relationships (SEIFERT 2009). In these cases, the delimitation of species should ideally be supported by different methods, in addition to morphological analysis, under the Integrative Taxonomy approach (see SCHLICK-STEINER & al. 2010).

This study aimed to provide new evidence about a probable sister-group relationship between *Crematogaster abstinens* and *C. pygmaea*, and to give new insight into the ecological significance of the highly polygynous and polydomous system found in *C. pygmaea*. We first investigated morphological (morphology of queens, workers and males), chemical (cuticular hydrocarbons profiles), behavioral (attractiveness of queens and of substrates chemically marked by queens, colony foundation, mating behavior) and colony-structure (nest architecture, queen number, nest number) traits in *C. abstinens* and *C. pygmaea*. We then compared those traits in the two species, in order to evaluate how they link or separate both species, and, in light of the obtained data, discuss a possible evolutionary scenario for the emergence of polygyny and polydomy in *C. pygmaea*. We also describe for the first time the morphology of queens and males, the nesting habit, and the colony structure in *C. abstinens*.

Material and methods

Study sites and voucher policy: All *Crematogaster pygmaea* or *C. abstinens* individuals (workers, queens, males) used in the experiments were from colonies located in areas in the municipality of Fortaleza (state of Ceará, northeastern Brazil) (3° 47' S, 38° 33' W), and nearby municipalities, or from colonies collected in the field (in the Forta-

leza region) and maintained in laboratory conditions, as detailed in the next sections.

Permits for field work and ant nests collections were issued by the Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis (IBAMA) (permit numbers 26226-1, 48053-1, 48053-2).

Voucher specimens of *Crematogaster pygmaea* and *C. abstinens* are deposited at the Myrmecological Collection of the Laboratório de Entomologia, Universidade Estadual do Ceará, in Fortaleza, CE, Brazil, and at the Entomological Collection Padre Jesus Santiago Moure, Universidade Federal do Paraná, Curitiba, PR, Brazil (DZUP).

Comparative morphological study of *Crematogaster abstinens* and *C. pygmaea*: The morphological study was carried out based on queens, males and workers from different colonies of *Crematogaster abstinens* and *C. pygmaea* aiming to cover all the eventual intra- and interspecific variation for both species. Since workers of these species present intracolonial allometry, observations were always made based on individuals of different size classes within the same colonies. This procedure was adopted considering the attenuations that size variation imposes on external anatomy characters such as coloring, sculpture and pilosity.

Observations of most conspicuous morphological characters were performed under a Leica stereomicroscope model S8 APO with indirect illumination (a LED ring coupled to the scope objective).

In order to observe the details of integument microsculpture, images of workers and queens of *Crematogaster abstinens* and *C. pygmaea* were obtained under an Oxford Vacuum Scanning Electron Microscope (SEM), model 6427. The specimens (a worker and a queen of each species) were cleaned by agitation in a solution of water and industrial detergent and sequentially dry-mounted in entomological triangles. The triangles were then fixed to double-sided carbon tapes in the stubs allocated to the inner chamber of the SEM, without previous metallization of samples. The entire procedure was performed at the Electronic Microscopy Center of UFPR, Curitiba, PR, Brazil.

Morphometric analyses: Workers of *Crematogaster pygmaea* and of *C. abstinens* were collected (n = 120 for each species) from six different colonies (20 workers per colony for each species) in order to perform three types of morphometric measurements: head width (HW), i.e., the maximum width behind the eyes in full-face view; pronotal width (PW), i.e., the maximum width of pronotum in dorsal view; Weber's length (WL), i.e., the maximum diagonal distance from base of the anterior slope of pronotum to metapleural lobe (HÖLLDOBLER & WILSON 1990, LONGINO 2003). All six *C. abstinens* colonies were from an area located on the campus of the Universidade Estadual do Ceará (UECE) (3°47' S, 38° 33' W), in Fortaleza; five *C. pygmaea* colonies were from areas located in the municipality of Fortaleza (two on the UECE campus, three in nearby areas), the sixth *C. pygmaea* colony was in the municipality of Caucaia (3° 40' S, 38° 45' W), some 26 km from Fortaleza. The same morphometric measurements (except for pronotal width) were performed with 10 queens (from different colonies) of each species.

Ten more workers and queens of each species, and from different colonies, were also used to measure the mesosoma length, width and height. The mean length, width and height were then used to calculate the mean mesosoma volume in both groups, for each species. Queen / worker mesosoma volume ratio was then computed in each species, as the mean queen mesosoma volume divided by the mean worker mesosoma volume.

Morphometric measurements were obtained using Leica LAS Interactive Measurements module and composite images generated by Leica LAS Montage / 3D-Viewer modules (Leica Application Suite – Version 4.5.0) and a Leica-DMC2900 digital camera attached to a Leica M205A stereomicroscope.

Chemical analysis of cuticular lipids: In each of 10 different colonies of *Crematogaster pygmaea* and *C. abstinens*, 50 workers were collected directly in the field while they were moving on trails or exiting a nest, and immediately placed in a small and clean polypropylene container. The day following their collection, they were killed by freezing and then immersed in 0.2 ml of hexane, in a 2 ml glass vial that was stored in the freezer until the extracts were analysed. Seven *C. pygmaea* colonies were from areas located in the municipality of Fortaleza (three on the campus of the Universidade Estadual do Ceará-UECE, four in nearby areas), with distances between the colonies ranging from ± 220 m to ± 9 km; two *C. pygmaea* colonies were located in the municipality of Eusébio (3° 52' S, 38° 25' W), some 17 km from Fortaleza; one *C. pygmaea* colony was located in the municipality of Caucaia (distance between the colonies of Eusébio and the colony of Caucaia: ± 42 km). All *C. abstinens* colonies were from areas located in the municipality of Fortaleza (eight on the campus of the UECE, two in nearby areas) with distances between the colonies ranging from ± 10 m to ± 3.5 km.

Cuticular lipids in the extracts were analysed by GC / MS using a Finnigan Polaris Q™ ion trap mass spectrometer interfaced to a Trace GC Ultra™ gas chromatograph equipped with a DB-5MS fused silica capillary column (30 m × 0.25 mm diameter × 0.25 µm film thickness) from J&W Scientific. The injection port and transfer line were set at 240 °C and 310 °C respectively. Following splitless injection of 2 µl of the sample, oven temperature was maintained at 50 °C for 1 minute, increased to 270 °C at 20 °C / min, maintained at 270 °C for 10 minutes, then increased to 310 °C at 3 °C / min, and then maintained at 310 °C for 5 minutes, using helium as carrier gas (1 ml / min). Qualitative and quantitative data were acquired by running the Thermo Finnigan Xcalibur™ 1.4. SR1 data system. Cuticular lipids were identified by analysing their mass spectra produced by electron impact (ion source operating at 200 °C with an ionization energy of 70eV, scan range m / z 40 - 700) and by comparing their GC retention times with those of reference compounds (Alkanes-mix 10: C10 to C35, Dr. Ehrenstorfer Gmbh). The proportions of the different compounds were obtained from peak areas. The GC / MS analyses were conducted at the Unit of Evolutionary Biology and Ecology of the Université Libre de Bruxelles (ULB) (Brussels – Belgium).

The Nei index (*I*) was calculated to estimate the degree of similarity between cuticular hydrocarbons (CHCs) profiles:

$$I = \frac{\sum^{n} X_i * Y_i}{\sqrt{\sum^{n} X_i^2 * \sum^{n} Y_i^2}}$$

where *n* = number of peaks, *Xi* = area (%) of peak *i* for sample x, and *Yi* = area (%) of peak *i* for sample *y*. *I* = 1 for two strictly identical profiles and *I* = 0 for two totally different profiles.

For each species, the CHCs whose relative peak area was less than 0.5% in all samples were excluded. A Nei index was calculated for 10 intraspecific comparisons, for each species, and for 10 interspecific comparisons. Each colony of one species was compared to two other colonies, randomly selected, of the same species. Likewise, each *Crematogaster pygmaea* colony was compared to a randomly selected *C. abstinens* colony (but excluding those that had already been selected). In total, each colony was therefore compared to three other colonies (2 conspecific and 1 heterospecific).

Attractiveness of queens to workers in *Crematogaster abstinens*: Attractiveness of *Crematogaster abstinens* queens was assessed with a procedure similar to that used by KELLER & PASSERA (1989a) and MARTINS SEGUNDO & al. (2012) to test the attractiveness of *Linepithema humile* (MAYR, 1868) and *C. pygmaea* queens respectively. The tested queen of *C. abstinens* was confined in a wire-mesh ring (2 cm in diameter, 5 mm high) placed in the center of a plastic Petri dish (8.5 cm in diameter) with Fluon®-coated sides. The bottom of the dish was covered with a white paper disc that was changed before each new experiment. The wire-mesh ring allowed the *C. abstinens* workers, but not the queen, to pass through. A piece of glass covering the wire-mesh ring prevented the queen from escaping.

An experiment began when 50 *Crematogaster abstinens* workers from the same colony as the queen to be tested were placed in the Petri dish. The workers were allowed to acclimatize for 5 minutes before a dummy (glass bead with roughly the same volume as a *C. abstinens* queen) (control) was introduced inside the wire-mesh ring (introduction of dummy = t_0). Three minutes after t_0, and then every 3 minutes for a total of 30 minutes, three types of measurements were made (total of 10 replicates for each type of measurement): number of workers on the dummy, number of workers around, and in physical contact with it (with legs and / or antennae), and number of other ants present inside the wire-mesh ring (including those on the underside of the piece of the glass covering the wire-mesh and on the inner wall of the wire-mesh).

After this first series of measurements, the dummy was removed and a queen (from the same colony as the workers that remained in the Petri dish) was introduced inside the wire-mesh ring (introduction of queen = t_0). Three minutes after t_0, and then every 3 minutes for a total of 30 minutes, 3 types of measurements were made (total of 10 replicates for each type of measurement): number of workers on the queen, number of workers around, and in physical contact with the queen (with legs and/or antennae), and number of other ants present inside the wire-mesh ring.

The attractiveness of the queen (or the dummy) in a specific experiment was recorded as the sum of two means: the mean of the 10 values for the number of ants on the queen (or the dummy) and the mean of these 10 values for the number of ants around, and in physical contact with, the queen (or the dummy). The mean of the 10 values for the number of other ants present inside the wire-mesh ring was considered as a measure of the zone of influence (a possible long-distance attractiveness) of the queen.

The experiment was repeated 47 times, with queens (n = 20) from 20 monogynous colonies maintained in laboratory conditions; some queens were used two to three times, with a time interval of at least two months between two consecutive use of the same queen.

Interspecific attractiveness to workers of substrates after exposure to queens: The attractiveness to workers of filter papers that remained in close and prolonged contact with *Crematogaster abstinens* or *C. pygmaea* queens was assessed with a procedure similar to that used by MARTINS SEGUNDO & al. (2012). Two circles (1.5 cm in diameter) were drawn in pencil on the disc of paper filter that covered the bottom of a plastic Petri dish (8.5 cm in diameter; Fluon®-coated sides), such that both were equidistant in relation to the center and the edge of the dish. In one circle, a *C. pygmaea* or *C. abstinens* queen was confined for a 24 h period, by means of a glass test tube (10 cm in length, 1.5 cm in diameter) placed vertically on the circle. The inside of the glass test tube was coated with Fluon® to a height of 2 cm to prevent the queens from climbing. The other circle contained no queen (control), but had a glass test tube placed vertically above it (inside coated with Fluon® to a height of 2 cm). The orientation of the two circles was changed by 90 degrees from one replicate to the other to avoid any potential influence of the laboratory environment (windows, artificial light, etc.).

After the 24-h period, the glass tubes and the queen were removed, and 50 *Crematogaster pygmaea* or *C. abstinens* were placed in the Petri dish and allowed to acclimatize for 5 minutes before beginning (= t_0) to record the number of ants present on each circle. The record was repeated every 3 minutes for 30 minutes (total of 10 measurements for each circle). The attractiveness of each circle was recorded as the mean of these 10 values.

Three experimental treatments were carried out: *Crematogaster abstinens* queen with *C. abstinens* workers (always from the same colony as the tested queen) (47 replicates), *C. abstinens* queen with *C. pygmaea* workers (47 replicates), *C. pygmaea* queen with *C. abstinens* workers (47 replicates). The experiments with *C. abstinens* queens were performed with queens (n = 20) from 20 monogynous colonies maintained in laboratory conditions; some queens were used two to three times, with a time interval of at least two months between two consecutive use of the same queen. The experiments with *C. pygmaea* queens were performed with queens from five polygynous colonies maintained in laboratory conditions, each containing 15 to 20 queens. The results for the experimental treatment "*C. pygmaea* queen with *C. pygmaea* workers" can be found in MARTINS SEGUNDO & al. (2012).

Two more experimental treatments were performed, using the same colonies and the same procedure as described above. However, each circle contained a queen: a *Crematogaster abstinens* queen in one circle, a *C. pygmaea* queen in the other. In one experimental treatment (ten replicates), 50 *C. abstinens* workers (from the same colony as the tested *C. abstinens* queen) were placed in the Petri dish after removal of the queens; in the other (ten replicates), 50 *C. pygmaea* workers (from the same colony as the tested *C. pygmaea* queen) were placed in the Petri dish after removal of the queens.

Colony structure and nest architecture: Between February and December of 2015, a period covering rainy season (January to May) and dry season (June to December), 12 *Crematogaster abstinens* colonies were excavated, in an area (± 200 × 30 meters) with dense herbaceous vegetation, located on the edge of an artificial lake, on the campus of the Universidade Estadual do Ceará (Fortaleza) (3° 47' S, 38 °33' W). Presence of colonies was first detected observing *C. abstinens* workers on extrafloral nectaries of herbaceous plants (mainly *Turnera subulata* J.E. SMITH). Each detected colony was then carefully examined, until a complete map of all visited plants, all trails and all orifices in the soil with in/out activity of workers was obtained. Sardine baits placed on the ground were also used to help detect orifices with ant activity.

After the mapping of a colony was completed, a trench initially ± 1 meter deep, 1 to 2 meters long and ± 1 meter wide was dug about 30 cm from the marked orifices with worker activity (sometimes, more than one trench had to be dug, depending on the spatial arrangement of the orifices). The soil of the trench wall was then carefully shaved away in the direction of the marked orifices until each tunnel and chamber was found. The dimensions, relative positions and depth of all tunnels and chambers were measured, photographed, and sketched. The population (brood, workers, female sexuals, and males) found in each chamber was recorded, and collected in order to perform a precise analysis of the size and composition of the whole adult population of the colony. The whole colony was then kept in a plastic box (20 cm × 20 cm and 7 cm high) with sides coated with Fluon®, at a constant temperature of 30 ± 2 ºC with a 12:12 L:D photoperiod, and with glass test tubes as nesting sites. The ants were fed ad libitum on 0.5 M sucrose solution and dead *Tenebrio molitor* LINNAEUS, 1758 larvae. Part of those laboratory colonies were used to assess the attractiveness of *Crematogaster abstinens* queens and the interspecific attractiveness of substrates after exposure to *C. abstinens* queens.

Using the same procedure as described above, 16 nests of *Crematogaster pygmaea* were excavated between August and November of 2014 (peak of dry season), in two polydomous colonies: two nests were from a colony located on the campus of the Universidade Estadual do Ceará, and that had been previously used to map *C. pygmaea* colonies and investigate nest architecture during rainy season (QUINET & al. 2009); the other nests (n = 14) were from a large colony located in the Municipal Garden of the city of Fortaleza (3° 48' S, 38° 32' W), distant some 3.5 km from the UECE.

Colony foundation strategies: In February 2015, at the beginning of rainy season, 20 gynes (here defined as unmated winged queens) of *Crematogaster pygmaea* were collected as they were leaving the home nest, just before engaging in a nuptial flight, in a large polydomous colony located in the Municipal Garden of the city of Fortaleza. In the same period, 60 gynes of *C. abstinens* were collected, together with males and workers, at the exact time when they were all leaving, in a very excited way, the nest of a colony located on the campus of the Universidade Estadual do Ceará, during what appeared to be a premating flight episode.

In the laboratory, each *Crematogaster pygmaea* gyne was placed in a plastic Petri dish (8.5 cm in diameter) with Fluon®-coated sides, together with five males from *C. pygmaea* colonies maintained in laboratory conditions. After copulation and dealation (wings shedding), each mated young queen was isolated in a glass test-tube (10 cm in length, 1 cm in diameter) that was provided with a water reservoir at the bottom of the tube, surrounded by a red plastic film, and whose open end was closed by a cotton plug. The glass test-tubes were kept in a room with a constant temperature (30 ± 2 °C) and a 12:12 L:D photoperiod, and were regularly checked to verify egg laying by queens.

The time necessary for the first larvae, the first pupae and the first adult workers to emerge in the young foundations was recorded. After the first adult workers emerged, each young foundation was placed in a plastic box (15 cm × 10 cm and 4 cm high; sides coated with Fluon®), in a room with constant temperature (30 ± 2 °C) and a 12:12 L:D photoperiod. The ants were fed ad libitum on sucrose solution (0.5 M) and dead *T. molitor* larvae. The number of workers and other adults (gynes, males) present in each young colony was then recorded every week for 16 months.

After unsuccessful attempts to obtain fertilization and dealation of the *Crematogaster abstinens* gynes, using the same system used for *C. pygmaea* gynes, the 60 *C. abstinens* gynes were placed together with the males and the workers collected in the field, in a plastic box (15 cm × 10 cm and 4 cm high; sides coated with Fluon®), in the same temperature, light, and food conditions as described above for the young *C. pygmaea* colonies. About two days later, 14 gynes were found dealated. Each one was isolated in the same way as described above for *C. pygmaea* dealated gynes, and the 14 young *C. abstinens* foundations / colonies were followed during more than one year (16 months; weekly the first week to the 47th week, and, then, at the 59th and the 68th week), with the same experimental procedure as described above for *C. pygmaea* foundations / colonies.

Mating and wings shedding behavior: In order to have more precise and quantified information about copulation and wing shedding (dealation) behavior in *Crematogaster pygmaea*, 18 gynes were collected in February 2016 (beginning of rainy season), in the large polydomous colony located in the Municipal Garden of the city of Fortaleza, as they were leaving the home nest, during a premating flight activity. In the laboratory, each *C. pygmaea* gyne was placed in a plastic Petri dish (8.5 cm in diameter; Fluon®-coated sides), together with five males from *C. pygmaea* colonies maintained in laboratory conditions. Four types of data were collected with each gyne: time elapsed between the beginning of the experiment (introduction of the gyne and males in the Petri dish) and the first copulation; number of copulations; duration of each copulation; time elapsed between the end of the first copulation and wings shedding (dealation).

The same experiment was carried out, in March 2016, with *Crematogaster abstinens* gynes (n = 10) and males produced in *C. abstinens* colonies kept in laboratory conditions (the five males placed in a Petri dish together with a gyne were always from a different nest than the gyne). In April, 2016, the experiment was renewed with 17 gynes produced in laboratory colonies.

Statistical analyses: Unpaired two-sample *t*-test (Student's test) was used to compare means of morphometric

measurements (HW, PW, WL) in workers and queens of the two ant species. Mann-Whitney U test was used to compare intraspecific Nei indexes and interspecific with intraspecific Nei indexes. According to the experimental context and the statistical conditions encountered, Wilcoxon matched-pairs signed-ranks test, Mann-Whitney U test, or repeated measures one-way ANOVA with post hoc Tukey test, was used to compare the means for queen (or dummy) or substrate attractiveness to workers. The statistical tests were run with the GraphPad Instat 3.10 software (GraphPad Software, San Diego California USA) and statistical significance was set at $\alpha = 0.05$.

Results

Comparative morphological study of *Crematogaster abstinens* and *C. pygmaea*: *Crematogaster abstinens* and *C. pygmaea* (Fig. 1) are morphologically closely related species since their workers share: (1) the median region of head dorsum smooth and shiny, with the cephalic lateral portions rugo-reticulated, and antennal fossae with concentric rugae; (2) head pilosity sparse, formed by short, stiff, appressed hairs, directed to the median longitudinal line of cephalic dorsum; (3) tibiae predominantly covered by appressed hairs; (4) petiole, in dorsal view, subquadrate, with convex lateral margins; (5) anteroventral projection of petiole absent; (6) in dorsal view, postpetiole considerably wide, exceeding the width of the petiole and with a poorly impressed median groove; (7) comparatively discrete allometry among workers, without wide variation in body size; and (8) coloration ranging from light brown to dark brown in both species (QUINET & al. 2009).

However, significant morphological differences are present. *Crematogaster pygmaea* differs from *C. abstinens* by the considerably shorter and triangular propodeal spines, only slightly longer than the maximum diameter of the propodeal spiracle opening; in comparison, *C. abstinens* presents long, spiniform propodeal spines, about twice as long as the maximum diameter of the propodeal spiracle opening (Fig. 1). Another significant difference between these species is the sculpture of the mesosoma, more specifically of the promesonotum. *Crematogaster abstinens* presents a strongly rugo-reticulated promesonotum, especially on the humeral portions and at the junction between the dorsal and lateral faces; in *C. pygmaea*, the promesonotum is predominantly smooth and shiny, only weakly reticulated on the posterior limits, near the junction with the metanotal suture. In addition, a discrete difference can be observed between workers of *C. abstinens* and *C. pygmaea* with respect to gaster pilosity. Workers of *C. abstinens* present the gaster with long and dense subdecumbent hairs, while in *C. pygmaea* the gastral hairs tend to be short, sparse and suberect; this difference is even more noticeable in small workers.

Among queens (Fig. 1), the most striking features separating both species are body size and coloring, which are more uniform and constant than observed in workers. Queens of *Crematogaster pygmaea* tend to be smaller (see the morphometric analyses results below) and lighter than the representatives of the same caste in *C. abstinens*. In addition, a more detailed comparison also shows a considerable difference in the pilosity distribution in queens of both species. Queens of *C. abstinens* are considerably hairier and sculptured, especially on head and waist (Fig. 1).

Males of both species (Fig. 1) probably present the highest observable morphological distinction, especially when we compare the individuals' body size. Although coloring and pilosity are relatively similar, males of *Crematogaster abstinens* tend to be almost twice as large as males of *C. pygmaea*, in addition to presenting a considerably more convex and prominent promesonotum (Fig. 1).

Morphometric analyses: Head width, pronotal width and Weber's length are on average 1.14 times higher in *Crematogaster abstinens* workers than in *C. pygmaea* workers (ratio of 1.15 for head width, 1.13 for pronotal width and Weber's length) (Student's *t*-test: $p < 0.0001$, for head width, pronotal width, and Weber's length); a similar but slightly higher ratio (1.21 on average) (1.20 for head width, 1.22 for Weber's length) was found with *C. abstinens* and *C. pygmaea* queens (Student's *t*-test: $p < 0.0001$, for head width, and Weber's length) (Tab. 1).

In both species, there is a large difference in size between queens and workers. In *Crematogaster abstinens*, head width and Weber's length are respectively 2.35 and 4.21 higher in queens; in *C. pygmaea*, similar ratios were found (2.27 for head width, 3.93 for Weber's length).

This high queen / worker dimorphism is also shown by the queen / worker mesosoma volume ratio. In *Crematogaster abstinens*, queen and worker mesosoma volume is respectively 4.165 ± 0.52 mm^3 (mean ± standard deviation, SD; n = 10) and 0.0665 ± 0.0155 mm^3 (mean ± SD; n = 10), giving a queen / worker mesosoma volume ratio of 62.65. In *C. pygmaea*, queen and worker mesosoma volume is respectively 2.42 ± 0.38 mm^3 (mean ± SD; n = 10) and 0.0359 ± 0.0093 mm^3 (mean ± SD; n = 10), giving a queen / worker mesosoma volume ratio of 67.32. The ratio between mean mesosoma volume in *C. abstinens* queens and mean mesosoma volume in *C. pygmaea* queens is 1.72 (1.85 with workers).

Chemical analysis of cuticular lipids: The cuticular hydrocarbon (CHCs) profiles of the two species show quantitative and qualitative differences (Fig. 2). Only seven CHCs are common to both species; 12 and two CHCs were detected only in *Crematogaster pygmaea* and *C. abstinens*, respectively (Fig. 2). CHCs profile of *C. abstinens* is much simpler than that of *C. pygmaea* and is almost completely included in the latter (Fig. 2). Only the last two CHCs are specific to *C. abstinens* (Fig. 2), with the penultimate being of the same family as those shared by the two species (i.e., a mixture of 11 + 13 monomethylalkanes) (the last CHC was unidentified). Another difference between the two species is that *C. pygmaea* has alkenes in its CHCs profile, but not *C. abstinens*.

The Nei indexes for intraspecific comparisons are very high and not significantly different between the two species (Mann-Whitney U test: $p = 0.143$): 0.977 ± 0.013 (range: 0.952-0.990) (mean ± SD; n = 10) for *Crematogaster pygmaea*; 0.963 ± 0.026 (range: 0.927-0.999) (mean ± SD; n = 10) for *C. abstinens*. The Nei indexes for interspecific comparisons are lower (mean ± SD: 0.757 ± 0.065; n = 10) (range: 0.654 - 0.864) than those obtained with intraspecific comparisons, and significantly different from them (Mann-Whitney U test: $p < 0.0001$ in both cases). Therefore, intraspecific profiles were statistically significantly more similar than interspecific profiles.

Attractiveness of queens to workers: Under laboratory conditions (Petri dish and wire-mesh device), *Crematogaster*

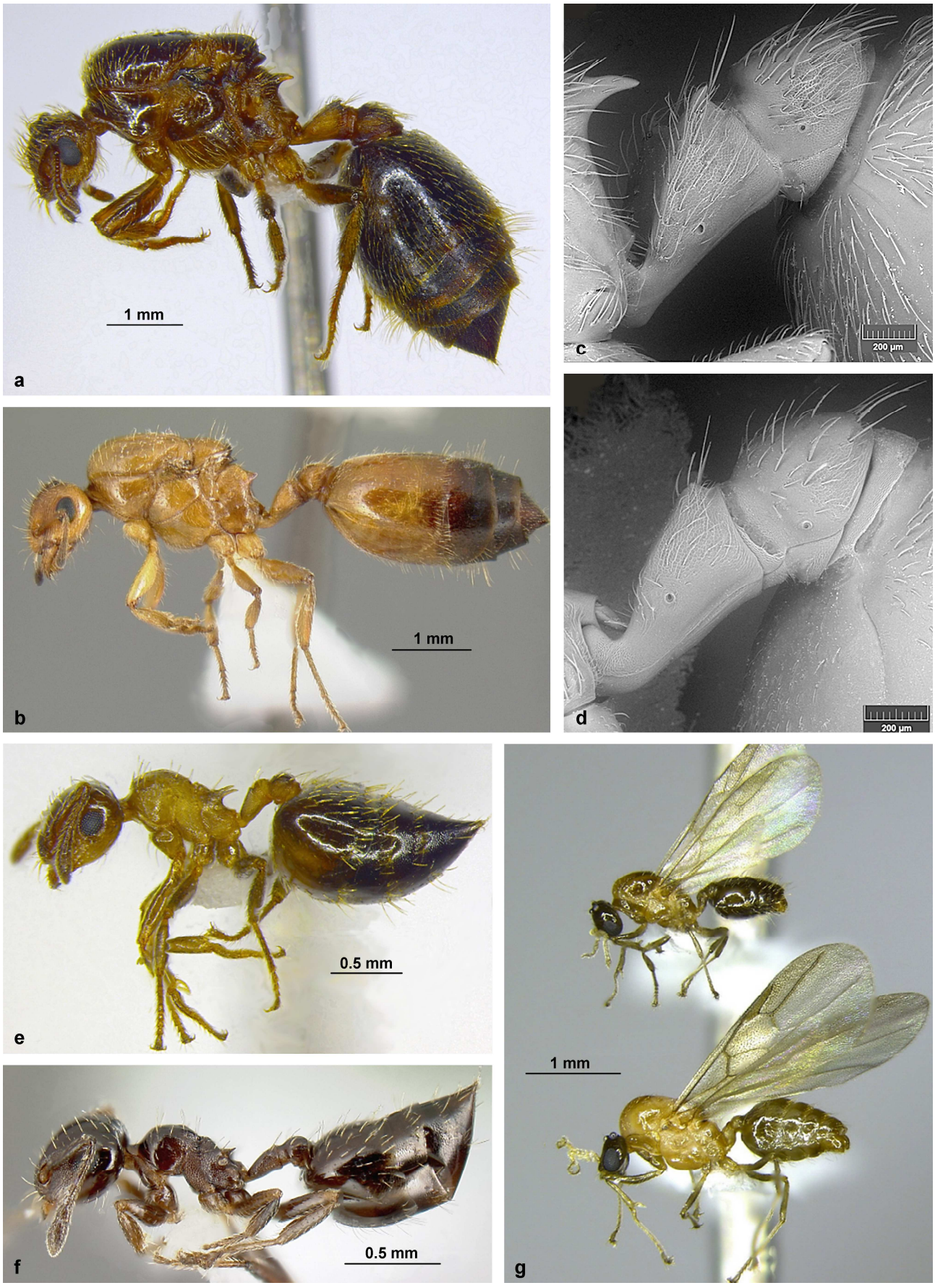

Fig. 1: Queens, workers, and males of *Crematogaster abstinens* and *C. pygmaea* in lateral view. (a) *C. abstinens* queen; (b) *C. pygmaea* queen; (c) *C. abstinens* queen, petiole; (d) *C. pygmaea* queen, petiole; (e) *C. abstinens* worker; (f) *C. pygmaea* worker; (g) *C. pygmaea abstinens* (upper) and *C. abstinens* (lower), males. Photos in (b) and (f): J. T. Longino.

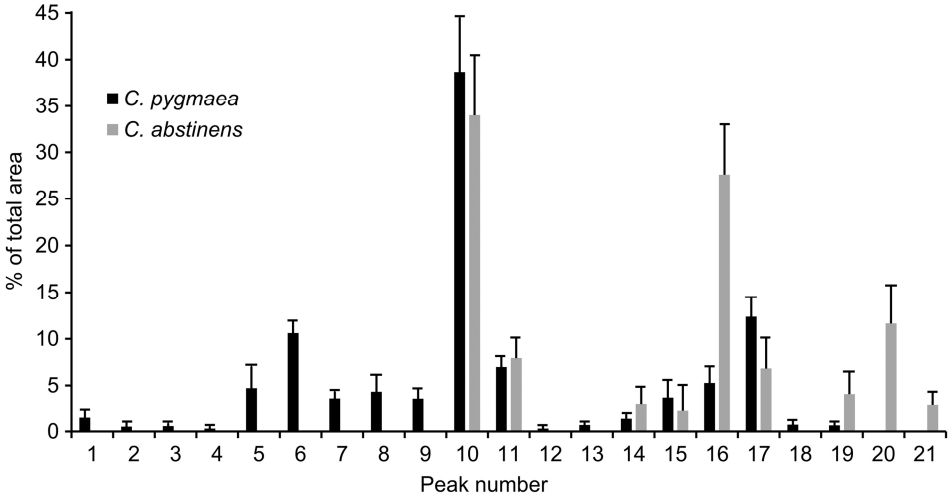

Fig. 2: Proportion of the different hydrocarbons (mean ± SD) identified in the cuticular profile of *Crematogaster pygmaea* and *C. abstinens* (n = 10 for each species). 1: xC23:1; 2: n-C23; 3: 9+11-MeC23; 4: n-C24; 5: xC25:1; 6: n-C25; 7: 11+13-MeC25; 8: 3-MeC25; 9: n-C26; 10: n-C27; 11: 11+13-MeC27; 12: 5-MeC27; 13: 3-MeC27; 14: n-C28; 15: 10+12-MeC28; 16:n-C29; 17: 11+13-MeC29; 18: unidentified CHC; 19: n-C31; 20: 11+13-MeC31; 21: unidentified CHC.

Tab. 1: Head width (HW), pronotal width (PW, only for workers), and Weber's length (WB) in workers (n = 120) and queens (n = 10) of *Crematogaster abstinens* and *C. pygmaea*.

	HW (mm), Mean ± SD	**PW (mm), Mean ± SD**	**WL (mm), Mean ± SD**
Crematogaster abstinens – workers	0.566 ± 0.037	0.397 ± 0.031	0.645 ± 0.047
Crematogaster pygmaea – workers	0.493 ± 0.024	0.349 ± 0.020	0.570 ± 0.035
Crematogaster abstinens **–** queens	1.335 ± 0.023	–	2.719 ± 0.067
Crematogaster pygmaea **–** queens	1.117 ± 0.017	–	2.230 ± 0.079

gaster abstinens queens' attractiveness to workers of its own colony (measured here as the number of workers on and around the queen, in direct physical contact with her) was higher (mean ± SD: 5.92 ± 2.13; n = 47) than that observed with a dummy (control) (mean ± SD: 0.15 ± 0.45; n = 47) ($p < 0.0001$; Wilcoxon test) (Fig. 3). It was also higher than that observed with *C. pygmaea* queens tested in exactly the same experimental conditions (with workers of its own colony) and device (mean ± SD: 3.73 ± 0.97; n = 10) (MARTINS SEGUNDO & al. 2012) ($p < 0.001$; Mann-Whitney U test) (Fig. 3).

The mean number of the other ants present in the wire-mesh device (i.e., the ants that were not on the queen or around and in physical contact with her) (mean ± SD: 21.71 ± 6.74; n = 47) where the *Crematogaster abstinens* queens were placed was also higher than that observed when a dummy was present in the device (mean ± SD: 9.16 ± 8.13; n = 47) ($p < 0.0001$; Wilcoxon test) (Fig. 3). It was also higher than the mean number of the other ants present in the wire-mesh device in similar experiments performed with *C. pygmaea* queens (mean ± SD: 6.93 ± 3.18; n = 10) (MARTINS SEGUNDO & al. 2012) ($p < 0.0001$; Mann-Whitney U test).

Interspecific attractiveness to workers of substrates after exposure to queens: The mean number of workers on a filter paper circle that had been in close contact with a queen for 24 h was much higher than that observed for the filter paper circle without queen (control), in all experimental treatments (*Crematogaster abstinens* queen with *C. abstinens* workers; *C. abstinens* queen with *C. pygmaea* workers; *C. pygmaea* queen with *C. abstinens* workers) ($p < 0.0001$; Wilcoxon test) (Fig. 4).

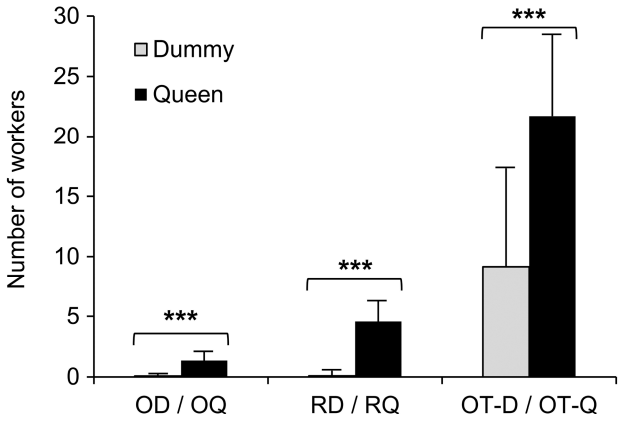

Fig. 3: Number of *Crematogaster abstinens* workers (mean ± SD; n = 47) on and around a *C. abstinens* queen (or a dummy). OD: on the dummy; OQ: on the queen; RD: around the dummy and in physical contact with it; RQ: around the queen and in physical contact with her; OT-D; other ants present in the wire-mesh ring device with dummy inside; OT-Q: other ants present in the wire-mesh ring device with queen inside. ***: $p < 0.0001$ (Wilcoxon test).

The mean number of workers on circle with queen was similar in the experimental treatments "*Crematogaster abstinens* queen with *C. pygmaea* workers" (mean ± SD: 8.16 ± 6.52; n = 47) and "*C. pygmaea* queen with *C. abstinens* workers" (mean ± SD: 7.55 ± 6.14; n = 47), but lower than that observed in the experimental treatment "C. *abstinens* queen with *C. abstinens* workers" (mean ± SD: 18.91 ±

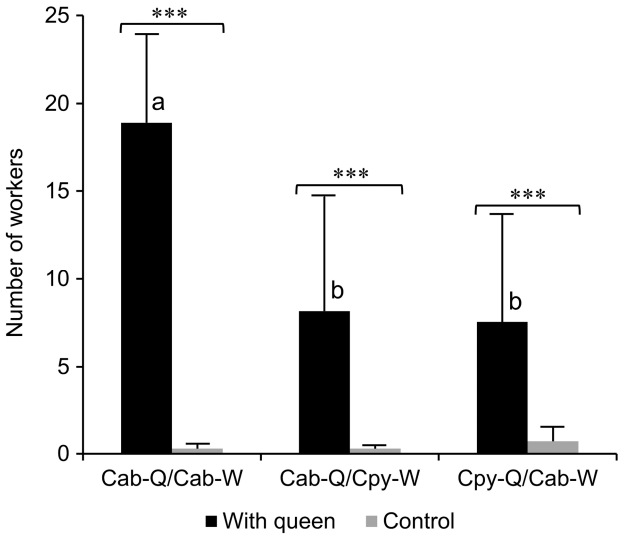

Fig. 4: Number of workers (mean ± SD; n = 47) on a paper circle where one queen had been held for 24 hours (With queen) and on another without a queen (Control) in three experimental treatments: queen of *Crematogaster abstinens* with workers of *C. abstinens* (Cab-Q/Cab-W); queen of *C. abstinens* with workers of *C. pygmaea* (Cab-Q/Cpy-W); queen of *C. pygmaea* with workers of *C. abstinens* (Cpy-Q/Cab-W). ***: $p < 0.0001$ (Wilcoxon test for the comparison of the means "With Queen" and "Control" in each experimental treatment. Different letters on "With queen" bars indicate statistically significant differences at $p < 0.05$ (one-way ANOVA with a post hoc Tukey test).

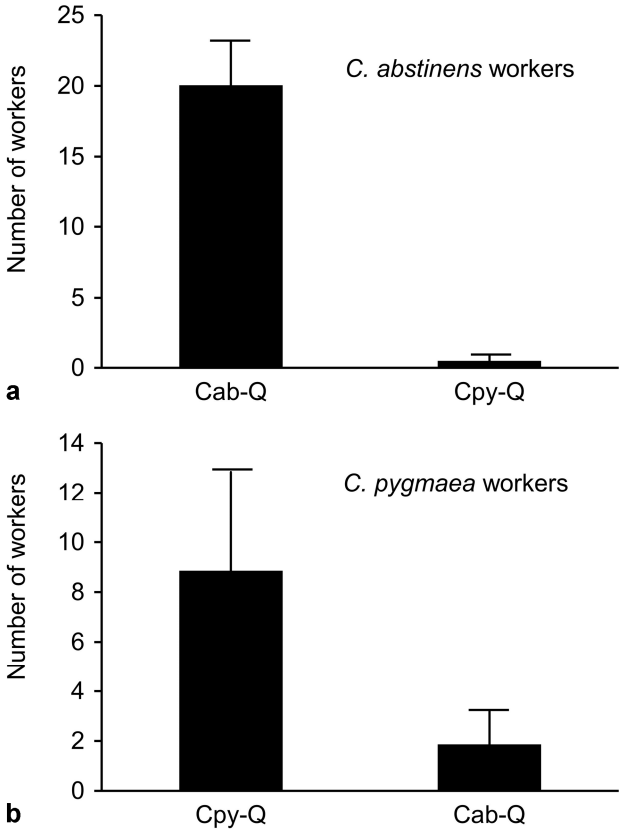

Fig. 5: Number of workers (mean ± SD; n = 10) on a paper circle where a queen of their own colony and species had been held for 24 hours and on another where a queen of the other species stayed. (a) with *Crematogaster abstinens* workers; (b) with *C. pygmaea* workers. $p < 0.0001$ (Wilcoxon test) for the comparison of the means in each case. Cab-Q: circle with *C. abstinens* queen; Cpy-Q: circle with *C. pygmaea* queen.

5.0; n = 47) (one-way ANOVA with post hoc Tukey test) (Fig. 4).

The mean number of *Crematogaster abstinens* workers on a filter paper circle that had been in close contact with a *C. abstinens* queen was higher than that observed in strictly similar experiments performed with *C. pygmaea* queens and workers (mean ± SD: 11.70 ± 5.49; n = 20) ($p < 0.0001$; Mann Whitney U test) (MARTINS SEGUNDO & al. 2012).

In the experiments where the *Crematogaster pygmaea* or *C. abstinens* workers had the choice between a paper circle that had been in close contact with a queen of their colony (and hence own species) and a circle that had been in close contact with a queen of the other species, the mean number of workers was always much higher on the circle with the queen of their own species (Fig. 5). Again, the mean number of *C. abstinens* workers on a filter paper circle that had been in close contact with a *C. abstinens* queen (mean ± SD: 20.04 ± 3.11; n = 10) was higher than the mean number of *C. pygmaea* workers on a filter paper circle that had been in close contact with a *C. pygmaea* queen (mean ± SD: 8.86 ± 3.98; n = 10) ($p < 0.0001$; Mann-Whitney U test).

Colony structure: The *Crematogaster abstinens* colonies were located near (some 100 meters) the place where *C. pygmaea* polydomous colonies had been mapped in previous work (QUINET & al. 2009). However, the vegetation structure found in the area with *C. abstinens* colonies (relatively dense herbaceous/shrubby vegetation) was different from that usually observed in areas where *C. pygmaea* colonies are found (open areas with sparse to moderately dense herbaceous vegetation, in man-made landscape) (QUINET & al. 2009, HAMIDI & al. 2012, CARLOS 2015, HAMIDI & al. 2017,). Such difference in the vegetation structure of areas occupied by *C. pygmaea* or *C. abstinens* colonies has been observed in several places of the Fortaleza region, although some overlapping of areas occupied by colonies of the two species was observed in some cases.

Eleven of the twelve excavated *Crematogaster abstinens* colonies had only one nest containing a single queen, brood, and a worker population whose size ranged from ± 300 to ± 2900 individuals (Tab. 2) (mean ± SD: 1291 ± 739 workers; n = 10). The ratio number of queen / 100 workers was more than ten times lower than that found in *C. pygmaea* (1 / 100) (QUINET & al. 2009). One colony (colony Nº 5 in Tab. 2) had two nests, each containing a queen, brood and a worker population whose size was between 300 and 370 individuals (Tab. 2).

All the nests (n = 13) consisted of a single simple hole in the ground and a single straight vertical tunnel, with four to 19 circular or elliptical chambers (mean ± SD: 10.8 ± 4.8; n = 13) (Fig. 6). Tunnel diameter was 2.14 ± 0.52 mm (mean ± SD; n = 199) and had a total length (from the hole in the ground until the bottom of the last chamber) ranging from 27 to 62 cm (mean ± SD: 48.2 ± 12.8 cm; n = 13) (Tab. 2). Diameter and height of chambers ranged

Tab. 2: Depth (from the nest entrance to the bottom of the deepest chamber) (D), number of chambers (NCh), queen number (NQ) and size of worker population (WPop) in excavated nests of *Crematogaster abstinens* colonies. Col. Nº: colony number; * worker population was incompletely collected.

Col. Nº	D (cm)	NCh	NQ	WPop
1	58.0	10	1	1375
2	47.5	18	1	881
3	40.0	10	1	2938
4	47.0	18	1	2039
5	26.5	5	1	369
5	29.5	4	1	316
6	38.5	9	1	1131
7	62.0	12	1	672
8	41.5	8	1	1445
9	68.0	9	1	160*
10	56.5	7	1	328
11	49.5	19	1	1047
12	62.0	11	1	1057

from 7 to 54 mm (mean ± SD: 26.4 ± 10.5; n = 344) and from 4 to 23 mm (mean ± SD: 8.6 ± 3.6; n = 172), respectively.

In addition to the nest(s), each colony had from three to 14 outstations (following terminology used by LANAN & al. (2011), here defined as simple holes in the ground with observed in/out movement of workers, but without tunnel underneath, or, in some cases, with a short tunnel (some centimeters in length) containing workers but without chamber and never containing brood or queen. Many of those outstations were found at the base (root) of gramineous plants apparently explored by the ants (in some cases, scale-insects were observed on the roots, with ants) (Fig. 7). In some colonies, one or two more elaborated structures (tunnel 10 to 30 cm in length, with some chambers), very similar to *Crematogaster abstinens* nests, were also found. However, they had no workers or brood, and probably were abandoned. The total area occupied by each *C. abstinens* colony ranged from ± 0.3 to ± 2.5 m^2 (Fig. 7).

Of the 16 *Crematogaster pygmaea* nests excavated in the 2014 dry season, three were not true nests: two of them consisted in only a short tunnel (7 and 17 mm in length), without chamber; the third was represented by only a hole in the ground, with a small space underneath. The other nests (n = 13) had a structure basically similar to that described in previous work (QUINET & al. 2009), for nests excavated in rainy season: a single vertical tunnel and several chambers arranged along it (Fig. 6). However, while the mean depth of nests in rainy season was ± 30 cm (QUINET & al. 2009), the depth of most of the nests excavated in dry season was greater than 50 cm and in many nests, it reached more than one meter (up to two meters for one of them) (Tab. 3, Fig. 6). A strong positive correlation was found between the length of the vertical tunnel

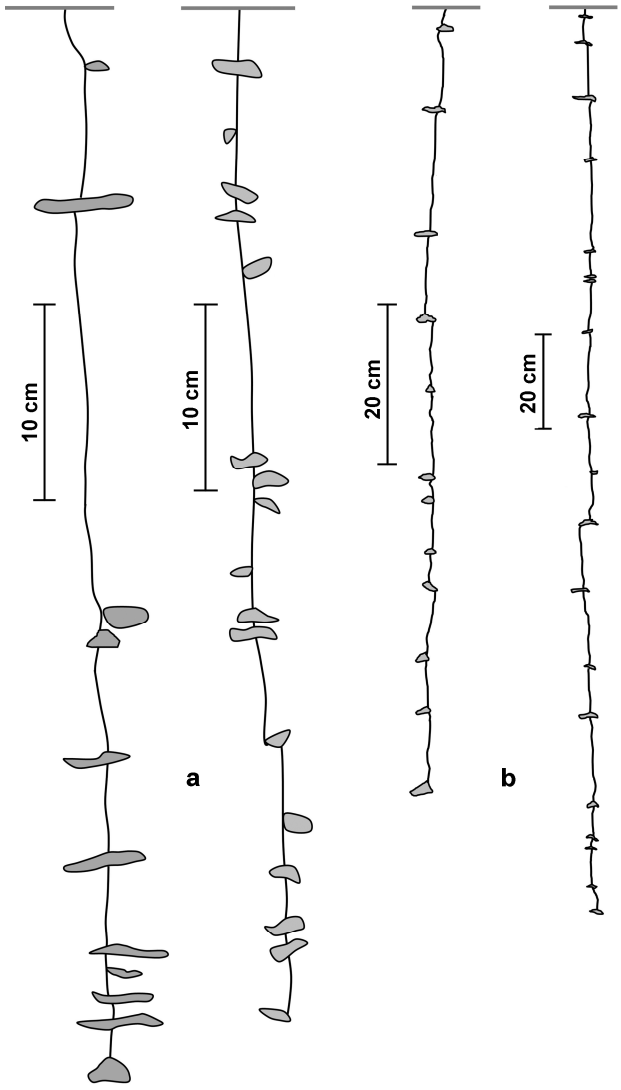

Fig. 6: Examples of *Crematogaster abstinens* (a) and *Crematogaster pygmaea* (b) nests, with vertical tunnel and chambers.

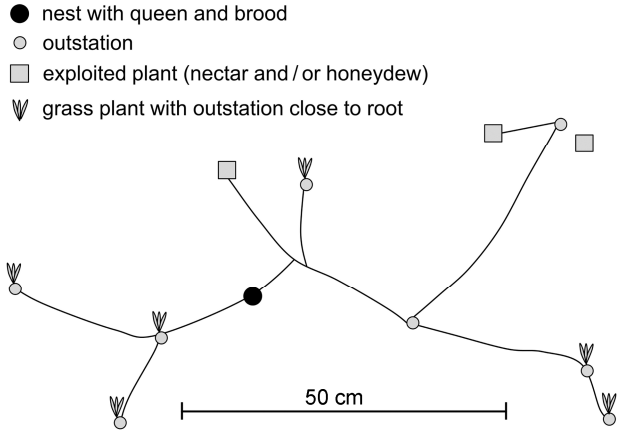

Fig. 7: Example of *Crematogaster abstinens* colony with nest, outstations and exploited plants.

(depth of the nests) and the number of chambers (from 1 to 18 circular or elliptical chambers, Tab. 3) found in the nests (Pearson product-moment correlation: r = 0.94, p < 0.0001; n = 13).

Tab. 3: Depth (from the nest entrance to the bottom of the deepest chamber) (D) and number of chambers (NCh) in 13 *Crematogaster pygmaea* nests excavated during the 2014 dry season.

Colony	D (cm)	NCh
1	202	18
2	135	18
3	127	14
4	120	11
5	114	13
6	112	10
7	102	12
9	82	7
10	43	5
11	25	3
10	14	2
12	7.5	4
13	5	1

Mean (± SD) diameter of tunnels was 1.83 ± 0.34 mm (n = 190). Diameter and height of chambers ranged from 9.4 to 38.8 mm (mean ± SD: 26.6 ± 9.6; n = 207) and from 4.2 to 8.9 mm (mean ± SD: 6.7 ± 2.9; n = 104), respectively. Those values are very similar to those found in *Crematogaster pygmaea* nests excavated in rainy season (QUINET & al. 2009) and in *C. abstinens* nests.

Colony foundation strategies: All *Crematogaster pygmaea* or *C. abstinens* dealated gynes began to lay eggs soon after they were isolated in glass-test tubes. However, queen survival in foundations (and afterwards young colonies) showed marked differences between the two species. While 10 (71.4%) of the initial *C. abstinens* foundations (n = 14) still had their queen alive 68 weeks after gynes fecundation (one queen died eight weeks after gynes fecundation, two more queens died 16 weeks after gynes fecundation, and a forth one died 32 weeks after gynes fecundation) (Fig. 8), only two (10%) of the initial *C. pygmaea* foundations (n = 20) still had their queen alive 71 weeks after gynes fecundation. In the other foundations / young colonies, the queens underwent a mortality that began as early as the first five weeks after fecundation of the gynes, and accentuated 37 weeks after fecundation (Fig. 8).

In *Crematogaster pygmaea* foundations, the first larvae, pupae and adult workers emerged three, six and seven weeks respectively after fecundation of the gynes; in *C. abstinens* foundations, the first larvae, pupae and adult workers emerged four, seven and eight weeks after fecundation, respectively.

In *Crematogaster pygmaea* foundations / young colonies whose queen survived for at least one year (52 weeks) (n = 8), the worker population grew exponentially over a period of 25 - 35 weeks, depending on the foundations, before rapidly declining in all young colonies (Fig. 9). Seventy-one weeks after fecundation of the gynes, all colonies were virtually extinct, except for one (Fig. 9). None of the young colonies produced adult gynes, nor gyne-destined brood (larvae or pupae). Only one colony began to produce males 36 weeks after fecundation of the gynes;

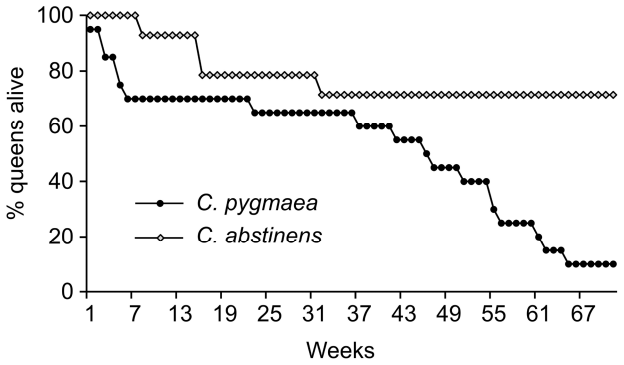

Fig. 8: Percentage of haplometrotic foundations / young colonies of *Crematogaster pygmaea* (n = 20) or *C. abstinens* (n = 16), with queen still alive one to 71 weeks after gynes fecundation.

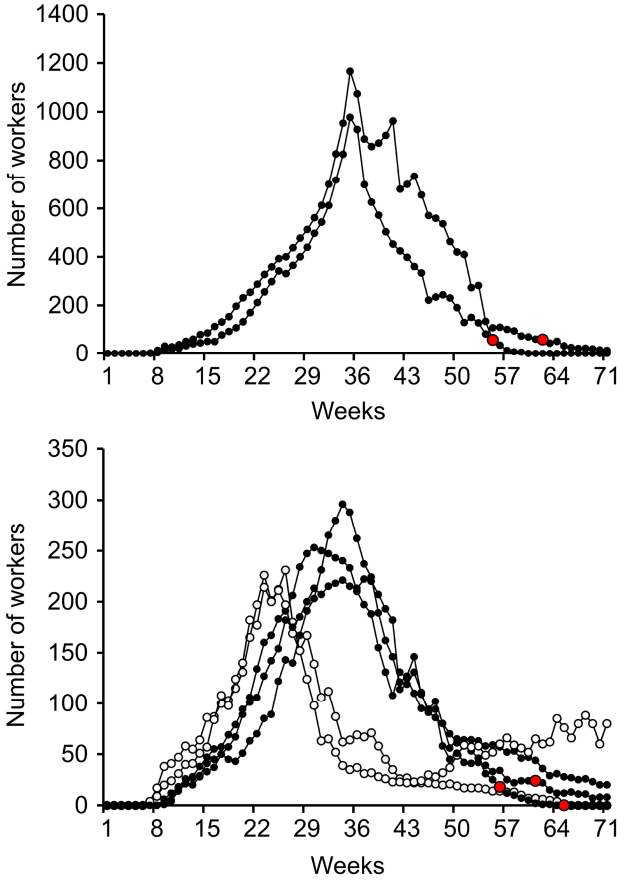

Fig. 9: Evolution with time of workers population in seven haplometrotic *Crematogaster pygmaea* foundations / young colonies in the 71 weeks period following gynes' fecundation (red circles: death of the queen).

the number of males reached a peak (107 males) after five more weeks, before suffering a rapid decline.

All the *Crematogaster abstinens* foundations / young colonies whose queen survived until the end of the observation period (68 weeks) (n = 10) showed a similar growth pattern of worker population: after the emergence of the first workers (eight weeks after fecundation), the worker population grew steadily until the end of the observation period (68 weeks), except for one (Fig. 10). At the end of the observation period, the mean (± SD) worker popula-

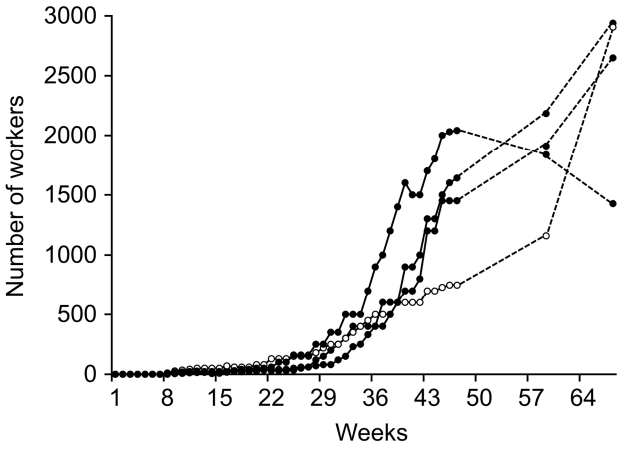

Fig. 10: Evolution with time of workers population in four haplometrotic *Crematogaster abstinens* foundations / young colonies in the 68 weeks period following gynes' fecundation (all queens were still alive after 68 weeks).

tion of colonies was 2349.6 ± 696.74 (n = 10), nearly twice the mean worker population observed in excavated nests, but nearly identical to the worker population found in the most populous excavated nests (Tab. 2). Larvae, pupae and adults of female sexuals emerged 37, 39 and 41 weeks after fecundation, respectively, in some (1 or 2) colonies. Fifty-nine weeks after fecundation, 60% of colonies (n = 6) were producing adult winged female sexuals (gynes) (one of them had 52 gynes). Thirty-one weeks after fecundation, adult males began to emerge in two colonies. Ten weeks later, 70% of colonies (n = 7) were producing males (one of them had 321 and 443 adult males 47 and 59 weeks after fecundation, respectively).

Mating and wing shedding behavior: All the *Crematogaster pygmaea* gynes (n = 18) individually placed in a Petri dish with five males were observed copulating with a male after a time ranging from 30 seconds to 67 minutes (mean ± SD: 27.2 ± 20.1 minutes; n = 18). The copulation time ranged from 11 to 46 minutes (mean ± SD: 23.06 ± 10.7 minutes; n = 18) and in most gynes (n = 14), the first copulation was followed by one, two or even three new copulations (in some cases, it was possible to verify that the new copulations were with different males). In most gynes, wing shedding (dealation) occurred from five to 122 minutes (mean ± SD: 32.85 ± 34.37 minutes; n = 13) after the end of the first copulation. However, four gynes shed their wings after more than 2.5 hours (more than five hours for one of them) after the end of the first copulation; one gyne never shed her wings. All the gynes that shed their wings (n = 17) began ovipositioning activity the day after dealation.

None of the 27 *Crematogaster abstinens* gynes individually placed in a Petri dish with five males was observed copulating with a male (observation period: 4 h), although each gyne was constantly surrounded by the males. In some cases, males climbed on the female gaster, and some repeatedly tried to copulate. However, all attempts by these males to establish a link between their genitalia and that of the female were unsuccessful. All the gynes were left in their Petri dish together with males (constantly renewed to replace those that died) for days, and all shed their wings seven to 68 days after they were placed together with males in a Petri dish. Each dealated gyne was isolated in a glass test-tube and all began oviposition activity the day after they were isolated. However, eggs were always flaccid and none developed into larva. Most gynes died one to five months after they were placed in isolation, in glass test-tube.

Discussion

Our study provides a convergent series of evidence supporting a close evolutionary relationship between *Crematogaster pygmaea* and *C. abstinens*.

The most robust line of evidence is the morphology. Although there is currently no phylogenetic proposal for the Neotropical species of *Crematogaster* (but see below), the morphological similarities observed between *C. abstinens* and *C. pygmaea* support an eventual sister-group relationship between these species, since the same level of morphological similarity is not found in other species related to them (LONGINO 2003). *Crematogaster abstinens* and *C. pygmaea* belong to a morphological complex distributed in the Neotropical Region that additionally includes the species *C. agnita* WHEELER, 1934, *C. obscurata* EMERY, 1895, *C. steinheili* FOREL, 1881, *C. victima* F. SMITH, 1858, and *C. victima cisplatinalis* MAYR, 1877 (LONGINO 2003). Although these species share morphological characteristics, no pair of species in this complex is as structurally similar as *C. abstinens* and *C. pygmaea*. In fact, this similarity is so pronounced that, until recently, *C. pygmaea* was considered a synonym of *C. abstinens* (LONGINO 2003). The species was then revived from the synonymy based on new data about the natural history of *C. pygmaea* populations in the state of Ceará (northeastern Brazil) and in morphological comparisons of specimens from the same populations (QUINET & al. 2009). The thorough morphological analyses carried out in this study make it clear that *C. pygmaea* and *C. abstinens* represent two distinct, though closely related lineages of *Crematogaster*.

In addition to the morphological evidence presented here, a project is in the final stages of preparing an internal phylogeny of the Neotropical species of *Crematogaster* using nuclear (Long-wave rhodopsin and Wingless) and mitochondrial (Cytochrome oxidase subunit I) markers. The preliminary results corroborate the sister-group relationship between *C. abstinens* and *C. pygmaea* with strong support (L.M. Pedraza-Hernández, pers. comm.). Regarding the phylogenetic position of this clade (*C. abstinens* + *C. pygmaea*), we presently prefer not to include these species in any group within the *Crematogaster* until Pedraza-Hernández and collaborators publish the conclusions, including an internal rearrangement, of their ongoing research.

Nesting habit and nest architecture are other traits shared by *Crematogaster pygmaea* and *C. abstinens*. Both are ground-dwelling species, a nesting habit relatively uncommon in *Crematogaster* ants (LONGINO 2003, HOSOISHI & al. 2010, BLAIMER 2012a), especially in the tropical and subtropical representatives of the group that are predominantly arboreal, nesting in dead branches, carton nests, under the bark (LONGINO 2003, BLAIMER 2012a), or even in *Nasutitermes* DUDLEY, 1890 nests (QUINET & al. 2005). The high similarity in nest architecture (the size, shape, arrangement, and dimensions of tunnels and chambers) in the two species is also a potential good indication of phylogenetic proximity, since detailed nest architecture of

ground-dwelling ant species is known to be species-specific (TSCHINKEL 2003).

The data obtained with the chemical analysis of cuticular hydrocarbons are more complex to interpret since the two CHC profiles show, at the same time, significant differences (compounds that are specific to either species, mostly in *Crematogaster pygmaea*; lack of alkenes in *C. abstinens*) and some degree of overlapping, with CHC profile of one species (*C. abstinens*) being almost completely included in that of the other (*C. pygmaea*). CHCs are known to be important cues for nestmate recognition in ant societies, and hence, for social cohesion and protection against homospecific or heterospecific intruders (D'ETTORRE & LENOIR 2009). They also provide other important signals that regulate reproductive activities, like fertility signals in reproductives (PEETERS & LIEBIG 2009). CHC profiles are generally species-specific and for that reason, are also thought to be promising tools for taxonomy, particularly in multidisciplinary approaches (LUCAS & al. 2002, D'ETTORE & LENOIR 2009, BAGNÈRES & WICKER-THOMAS 2010, BERVILLE & al. 2013, GUILLEM & al. 2016). Such approach has been successfully used to discriminate cryptic species or different species in a species complex, as in the *Pachycondyla villosa* (FABRICIUS, 1804) species complex (LUCAS & al. 2002), or Mediterranean species of *Tapinoma* FÖRSTER, 1850 (BERVILLE & al. 2013). The significant qualitative differences between *C. abstinens* and *C. pygmaea* CHC profiles, together with the high intraspecific similarity of CHC profiles, therefore support the morphologically based conclusion that *C. abstinens* and *C. pygmaea* are distinct species. On the other hand, the overlapping of a significant part of the two CHC profiles could explain the interspecific attractiveness observed with substrates chemically marked by queens of *C. pygmaea* or *C. abstinens*. Nevertheless, when workers have the choice between signals from a queen of their own species and colony, and signals from a queen of the other species, they choose the homospecific signal, showing that each CHC (or other cuticular compounds) profile also has specific elements allowing the workers to correctly discriminate signals from the two species. However, the interspecific attractiveness could be an additional evidence of the phylogenetic proximity between the two species, although, so far, there is no clear way to infer phylogenetic relationship between ant species from the comparison of their CHC profiles (MARTIN & al. 2008, WILGENBURG & al. 2011, GUILLEM & al. 2016).

Our study also revealed other important differences between the two species. One of them is the type of habitat where the two species are found: areas with relatively dense herbaceous / shrubby vegetation (at least in Fortaleza region), and dry / wet forests (LONGINO 2003) for *Crematogaster abstinens*; open areas with sparse to moderately dense herbaceous vegetation, in man-made landscape for *C. pygmaea* (QUINET & al. 2009). However, the most important difference between the two species is the colonial structure: *C. pygmaea* forms large highly polygynous and polydomous colonies (QUINET & al. 2009, CARLOS 2015), while *C. abstinens* colonies are small, monogynous and monodomous.

The clear-cut difference in queen number between the two species (strict monogyny in *Crematogaster abstinens*, high polygyny in *C. pygmaea*) could explain the observed differences in attractiveness of the queens and of substrates chemically marked by queens. In both species, queens are attractive to the workers, as are filter paper substrate that have remained in close and prolonged contact with queens. However, attractiveness obtained with *C. abstinens* queens was, in both cases, higher than that obtained with *C. pygmaea* queens. This result is consistent with the observation that queens of monogynous species are generally much more attractive than queens of polygynous species (KELLER 1988, KELLER & PASSERA 1989a). In *C. pygmaea*, it has been shown that the queen attractiveness is context-dependent (i.e., increasing with increasing degree of potential danger for the queen) and that it is involved in queen defense (MARTINS SEGUNDO & al. 2012). A similar function could exist for *C. abstinens* queens.

Monogyny / monodomy in *Crematogaster abstinens* and polygyny / polydomy in *C. pygmaea* probably represent adaptations to different types of habitat. One of the ecological determinants proposed to explain the evolution of polygyny is the patchy distribution of suitable habitats, and the associated high costs linked to long-range dispersal and independent colony founding by young queens (HÖLLDOBLER &WILSON 1977, HERBERS 1993, KELLER 1995, HEINZE & FOITZIK 2009). With regard to polydomy, one of the most preferred hypothesis is that it would represent a way to increase the rate of capture (discovery and exploitation) of resources (food and/or nest sites) in heterogeneous environment (patchy food distribution, for example), through decentralized central-place foraging behavior (DEBOUT & al. 2007, ROBINSON 2014). In contrast to *C. abstinens*, *C. pygmaea* lives in an anthropogenic (urban or semi-urban) habitat that consists of small to medium sized habitat patches (open areas with sparse herbaceous vegetation), isolated by much larger areas with trees and/or shrub vegetation (QUINET & al. 2009). Furthermore, this habitat is exposed to harsh climatic conditions (semiarid climate of northeastern Brazil, with rainfall concentrated in three consecutive months of the year and dry season during the rest of the year) that can add more instability to the anthropogenic habitat patches where *C. pygmaea* colonies are found. In C. *pygmaea*, polygyny and polydomy could therefore represent a combined evolutionary response to efficiently explore and rapidly saturate (through polygyny and a dynamic and flexible polydomous system) habitats that are unstable and subject to strong seasonality (CARLOS 2015, HAMIDI & al. 2017).

In previous studies (HAMIDI & al. 2017), it has been showed that *Crematogaster pygmaea* probably has a dual dispersal strategy: a long-range dispersal strategy where part of the gynes (unmated winged female sexuals) produced at the beginning of rainy season engage in mating flights followed by independent colony foundation, and a short-range dispersal strategy where the other gynes (may be most of them) mate in the parental colony and are readopted. In ants, swarming is thought to be an important trigger to initiate mating behavior (BAER 2011). The extreme ease with which *C. pygmaea* gynes mate and shed their wings after fecundation in artificial devices (Petri dishes), without swarming, could be therefore a good indication that intracolonial (and even intranidal) mating is a common behavior in that species. The long-range dispersal strategy would be a reproductive strategy allowing dispersers to enter new suitable patches to found new col-

onies, and, therefore, a way to escape from habitat instability (ex: deteriorating habitat) or saturation, while the short-range dispersal strategy would be part of a foraging strategy (seasonal polydomy) allowing the colony to expand the nests network when food sources are more abundant and when need for food sources is high (more brood to feed) (HAMIDI & al. 2017).

This dual dispersal strategy could explain the apparent paradox of a highly polygynous species where queens retain all the morphological features generally found in species with monogynous colony structure and independent founding strategy, like the voluminous mesosoma (enlarged wing muscles) and the high degree of dimorphism between queens and workers (metabolic reserves to sustain independent foundation) (KELLER & PASSERA 1989b, KELLER 1991, STILLE 1996, PEETERS & ITO 2001, PEETERS & MOLET 2009). However, the collapse of haplometrotic foundations / young colonies after an initial growth phase and the consequent inability of these colonies to reach the reproductive stage suggest that in *Crematogaster pygmaea*, independent foundation could be through pleiometrosis leading to primary polygyny. Our data on foundations also suggest that *C. pygmaea* queens have a short life span of about one year. This relationship between queens' short life span and polygyny was observed in other species (KELLER & PASSERA 1990), particularly in highly polygynous species (PASSERA 1994). Finally, our finding that *C. pygmaea* nests are much deeper in dry season, when polydomous colonies undergo a strong reduction in nest number (QUINET & al. 2009, CARLOS 2015), suggests that those deep nests could serve as refuges for the queens that survive in the dry season and to produce new female sexuals that would be available at the beginning of the next rainy season.

The data obtained with *Crematogaster abstinens* are all in line with mating flight (long-range dispersal strategy), mating away from the parental colony and claustral independent colony foundation strategy: high queen / worker dimorphism, high success for independent foundation in haplometrotic conditions, monogyny, and impossibility to obtain gyne fecundation in laboratory conditions (HÖLLDOBLER & WILSON 1990, KELLER 1991, STILLE 1996, PEETERS & ITO 2001, PEETERS & MOLET 2009).

In ants, monogyny and monodomy are considered to be the ancestral states of colony structure (DEBOUT & al. 2007, STEINER & al. 2009, BOULAY & al. 2014). In the light of the results obtained in this study, our hypothesis is that the polygynous and polydomous colony structure found in *Crematogaster pygmaea* is a derived condition of the monogynous and monodomous colony structure found in *C. abstinens*, and possibly in other related species.

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