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Behavioral Specialization of Workers of the Leafcutter Ant *Acromyrmex octospinosus* (Hymenoptera: Formicidae) with an External Bacterial Layer

Johannes-Paul Fladerer · **Franz Bucar**

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Abstract Leafcutter ants live in symbiosis with fungi they grow for food and different bacteria they use for protection against fungal parasites. Some workers are covered with a layer of bacteria like *Pseudonocardia*, but the behavior of these workers has been the subject of little research. In order to obtain a clearer understanding of this phenomenon in *Acromyrmex octospinosus*, two experiments were conducted with majors, minors, workers with little bacterial layer and workers with a well-developed bacterial layer. In a first step, scan sampling was used to distinguish the preferred location of workers with bacterial coats, compared to the preferences of their nestmates. Finally, 21 behavior patterns of workers with a bacterial layer on their integument were investigated through focal observations and compared to the behavior of their nestmates. Our findings suggest that workers covered with a high amount of *Pseudonocardia* differ from their sisters in their preference of location, in that they significantly prefer the bottom part of the nest and cannot be found outside the fungus chamber in the feeding area or the waste area. Additionally, these ants show more fungus grooming and are groomed by nestmates more often than the other workers. These results suggest that workers with an external bacterial layer evolved behavioral specialization.

Keywords Behavior · *Pseudonocardia* · bacterial layer · preference of location

Introduction

For more than 50 million years, a subclade of ants in the subtribe Attini has lived in a symbiosis with basidiomycetous fungi of the families Agaricaceae and Pterulaceae (Wheeler 1907; Weber 1958, 1966; Chapela et al. 1994; Mehdiabadi and Schultz 2010; Schultz et al. 2015). Among fungus-farming ants, the leafcutters, a small group of species in the genera *Atta* and *Acromyrmex*, collect leaves from many different plants and transport them into their nest to cultivate the fungi with it (De Fine Licht et al. 2010; Mehdiabadi and Schultz 2010; Hölldobler and Wilson 2011). Furthermore, the fungus-farming ants establish constant and optimal living conditions for the fungi in their nest chambers. In return, the fungi produce special bodies, called gongylidia, which are eaten by the ants (Quinlan and Cherrett 1979; Hölldobler and Wilson 2013; De Fine Licht and Boomsma 2014). Despite the fact that recombination takes place in the symbiotic fungi (Mikheyev et al. 2006), the fungi are mainly spread by the ants with little sexual reproduction (Currie et al. 1999; Hölldobler and Wilson 2011; Meirelles et al.

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J.-P. Fladerer (✉) · F. Bucar
Institute of Pharmaceutical Sciences, Karl-Franzens-Universität Graz, Universitätsplatz 4, 8010 Graz, Austria
e-mail: johannes.fladerer@uni-graz.at

2016). This lack of sexual reproduction likely contributes to a parasitic burden, such as infection by pathogens like *Escovopsis* (Muchovej and Della Lucia 1990; Dhodary et al. 2018). Besides behavioral (Nilsson-Møller et al. 2018) and chemical defenses (Ugelvig and Cremer 2007; Walker and Hughes 2009), like the production of many different acids and alcohols in Dufour´s gland and metapleural gland (Maschwitz et al. 1970; Beattie et al. 1986; Bot et al. 2002; Poulsen et al. 2002; Fernández-Marín et al. 2006, 2009; Yek and Mueller 2011; Worsley et al. 2018), a symbiosis with a third kind of symbionts has evolved as a solution to the fungi´s problem of becoming infected (Ortius-Lechner et al. 2000; Currie 2001b; Currie et al. 2003). Some bacteria living on the workers´ integument produce antimicrobials that inhibit pathogen growth (Poulsen et al. 2002; van Borm et al. 2002; Currie et al. 2003; Barke et al. 2010; Scheuring and Yu 2012; Andersen et al. 2015; Boya et al. 2017; Batey et al. 2020). For ants in the genus *Acromyrmex*, bacteria of the genus *Pseudonocardia* are known to be responsible for the production of such antimicrobials (Currie et al. 2003; Cafaro and Currie 2005; Sen et al. 2009; Poulsen et al. 2010; Caporaso et al. 2012; Holmes et al. 2016). The production of antimicrobials in combination with mechanical cleaning and the secretion of acids and alcohols from glands is an effective way to create an environment well-adapted to the fungi (Ortius-Lechner et al. 2000; Currie et al. 2006; Abramowski et al. 2011; Hölldobler and Wilson 2011).

For *A. octospinosus* two castes of workers are described (Wetterer 1999) differing in their behavioral defense against fungus-garden parasites (Abramowski et al. 2011). Some major workers of the leafcutter ant *Acromyrmex octospinosus* (Reich 1793) have a bacterial layer on the propleural plate or their integument is completely covered by bacteria. There is a specific time after eclosion when workers become inhabited by symbiotic bacteria (Poulsen et al. 2002, 2003), but at every age, most of the minors and most of the majors do not possess such bacterial layers, or it is reduced to a small, nearly invisible area on their propleural plate (Currie et al. 2003; Poulsen et al. 2003). Differences in external microbiota might lead to a specific function within the colony and therefore to a specialization in behavior. Behavioral differences between workers with a bacterial layer on the propleural plate and workers with a completely covered integument can be a result of the ants' temporal polyethism as the bacterial layer changes over time. Besides the bacterial layer could be a main factor which accounts for any differences in the ants' behavioral pattern as these two types of workers occur at the same age.

In order to investigate if workers with a bacterial layer have any behavioral differences or a behavioral specialization, two behavioral experiments were performed through scan sampling and focal observations. The aim of these experiments was to determine the location and behavior of various types of workers inside the colony, distinguishing between minor workers (minors), major workers without external bacterial layer (majors), majors with external microbiota on their propleural plate (BacI), and majors with such microbiota on their whole integument (BacII). Thus, we separated the major caste into three groups (majors, BacI and BacII). A representative picture of the different groups tested is given in the supplemental material (SFig. 1).

An understanding of the distribution and location of these workers should give an insight into their role within the colony. Ants protecting the fungi from pathogens, for example, should be located in or on the fungal garden, especially on the old fungi. Therefore, our experiment builds on previous research showing that workers covered with external microbiota could be found on the bottom of the nest more often than on the top (Poulsen et al. 2002). In our research, we measured the percentage of the different types of workers on the upper portion of the fungal garden where new fungus grows, on the bottom portion of the nest, in the feeding area and in the waste area outside the nest. Thus, our work deepens the knowledge gained by (Poulsen et al. 2002) as more areas of interest are defined and observed. Furthermore, the composition of worker types in highly specific areas outside the nest, like the feeding area and the waste area, can give new insights into specific functions of these worker types.

Additionally, investigating 21 behavioral patterns of those different types of workers inside the nest allows us to clearly associate them with special functions. Behavioral patterns like grooming, cutting, the treatment of brood and fungi and others by minors and majors have widely been studied in the past, and their temporal polyethism has also been thoroughly investigated (Hölldobler and Wilson 1990). After a first time inside the nest, many major workers go out to cut and carry leaves. Some stay inside the nest to cut the leaves into smaller pieces or to dispose of the waste. Minor workers are likely to stay inside the nest for their whole life and are associated with the treatment of the brood and the fungi (Hölldobler and Wilson 2011, 2013). In contrast to that, there has been little research concerning the behavior of workers covered with *Pseudonocardia*. Therefore, we investigated the behavior of majors with bacterial cover in more detail and compared it with the behavior of majors without such bacteria and with the behavior of minors. Thus, this work gives new insights into the behavioral specialization of workers with an external layer of symbiotic bacteria on their integument by combining their preference of location with behavioral pattern.

Material and Methods

Ants of the species *A. octospinosus* were collected in Colombia in 2014 and subsequently kept in sets of three formicaria linked with transparent hoses under laboratory conditions. One formicarium served as feeding arena, the middle formicarium contained the nest and the third one enabled the ants to dispose of waste. The temperature and the humidity were regulated by a thermo hygrometer. The temperature was between 20 and 25 °C and the humidity in the formicarium containing the nest was over 75%. The ants were fed with leaves of *Prunus fruticosus agg.*, *Vitis vinifera*, *Rosa sp.*, *Hedera helix* and *Acer plantanoides* ad libitum. Voucher specimens of the ants are deposited at the Karl-Franzens University of Graz. For the experiments, we used three colonies. The colony size was between 5 000 and 10 000 individuals. *A. octospinosus* was identified by the general description of (Reich 1793; Weber 1966; Currie et al. 2003) and by comparison with specimen CASENT0246632, CASENT0922028 and FMNHINS0000046890. A scheme of the experimental setup is given in supplemental material (SFig. 2).

Preference of Location

To find differences between major workers (major), minor workers (minor), ants with external microbiota on their ventral propleural plate (BacI) and ants with such microbiota on their whole body (BacII), the location preference of the ants was investigated by scan sampling. To investigate the location preference of these four worker types, we defined four areas of interest where our scans took place. The first area was a part of the transparent hose which the ants had to go through to reach plants to harvest. Because of the hose's transparency and the fact that the ants had to pass this area, one by one the different types of workers could be distinguished and counted easily. The second and third areas were the upper surface of the nest where young fungus grows, and the bottom side of the nest containing the old fungus. The last area we scanned was the formicarium containing the waste. Three replicas of the experiment were performed. Each colony was kept in its own set of formicaria. For each scan, 40 workers of each area were scanned at random and the number of major, minor, BacI and BacII per 40 workers per area scanned was noted. Twenty scans were made for each area in each colony. Each area was scanned only once a day. Raw data are given in supplemental material (STab1).

Behavior Inside the Nest

The behavior of the workers inside the nest was investigated by focal observations. Therefore, eight minor workers (minor), eight major workers (major), eight workers with bacteria on the propleural plate (BacI) and eight workers completely covered with bacteria (BacII) were observed in all three replica. The worker types could be distinguished without marking the ants, as in the formicaria used (the nest formicarium), the ants could be observed from different directions. Their propleural plate was therefore visible. Their behavior was recorded every thirty seconds for six hundred seconds. We distinguished between the behavior pattern given in Table 1.

The first nine behavioral pattern are well described by (Garrett et al. 2016). The other behaviors and their general functions have been described in previous research (Hölldobler and Wilson 1990; Beshers and Fewell 2001; Hart et al. 2002; Seifert 2007; Richard and Errard 2009).

After observation, the ants which had been observed were removed to avoid pseudoreplication. Raw data are given in supplemental material (STab2).

Table 1 Description of behavioral patterns observed in the fungus garden

Behavior	Description of behavior
Holding leaves	Ants are stabilizing a leaf fragment with mandibles.
Licking leaves	Ants make contact with leaf fragment with glossa and palps.
Scraping leaves	Making contact with the leaf fragments with mandibles while licking.
Cutting leaves	Reducing the size of leaf fragments by cutting with mandibles.
Puncturing leaves	Repeatedly piercing both sides of leaf fragments with mandibles.
Adding faeces	Depositing abdominal emissions onto leaf fragments.
Caching fragments	Caching leaf fragments. Often at nest entrance.
Inserting fragments	Inserting fragments into comb structure.
Inoculating fragments	Planting fungus into or between leaf fragments in the comb.
Grooming fungus	Ants are grooming fungus.
Removing fungus	Removing fragments of fungus from the comb.
Self-grooming	Equal autogrooming. An ant is grooming itself.
Grooming active	The ant under observation actively grooms another ant.
Grooming passive papassivepassive	Another ant grooms the ant under observation.
Trophallaxis	Ants are feeding each other (active and passive).
Treatment of eggs	Every activity associated with the eggs.
Treatment of larvae	Every activity associated with the larvae.
Treatment of pupae	Every activity associated with the pupae.
Immobility	Staying immobile without interaction with other ants.
Tactile communication	Communicating with antennal contact.
Walking	Walking without demonstrating other behavior.

Statistics

The data on the location preference of the ants and their behavior inside the nest was prepared for statistical analysis in SPSS 25. To compare worker types, a chi-square test (χ^2) was used. For direct comparison of pairs, a two-tailed Fisher´s exact test (FET) was performed. The α level was set at 0.05. Data in the text is given as mean ± standard deviation. In case of preference of location means are given as percentage. For differences in behavioral pattern means are given as arithmetic means of behavioral counts per ant.

Results

Preference of Location

Our behavioral experiments focusing on the location preference of the workers show a strong difference between the types of workers observed (χ^2 (9, $N=3200$) = 994.95 $p<0.05$) while there was no significant difference between the three colonies ($p>0.05$). Whereas on the old fungi, a percentage of 8.25 ± 3.8% belong to BacII and 9.25 ± 4.48% belong to BacI, these percentages decreased significantly to 2.75 ± 2.49% (FET, $p<0.05$) and 4.75 ± 3.7% (FET, $p<0.05$) for BacII and BacI on the growing zone. Outside the nest in the feeding area and on the waste, there are no such workers (Fig. 1). Minors also have a strong association with the nest, as they are nearly absent in the feeding area (<1%) but made up 53.63 ± 8.6% on the old fungi, 36.88 ± 7.5% on the growing zone and 53 ± 7.9% in the formicarium containing the waste.

Differences in Behavioral Pattern

Across all colonies, the worker types differed in their behavioral pattern (χ^2 (60, $N=1673$) = 1019.5

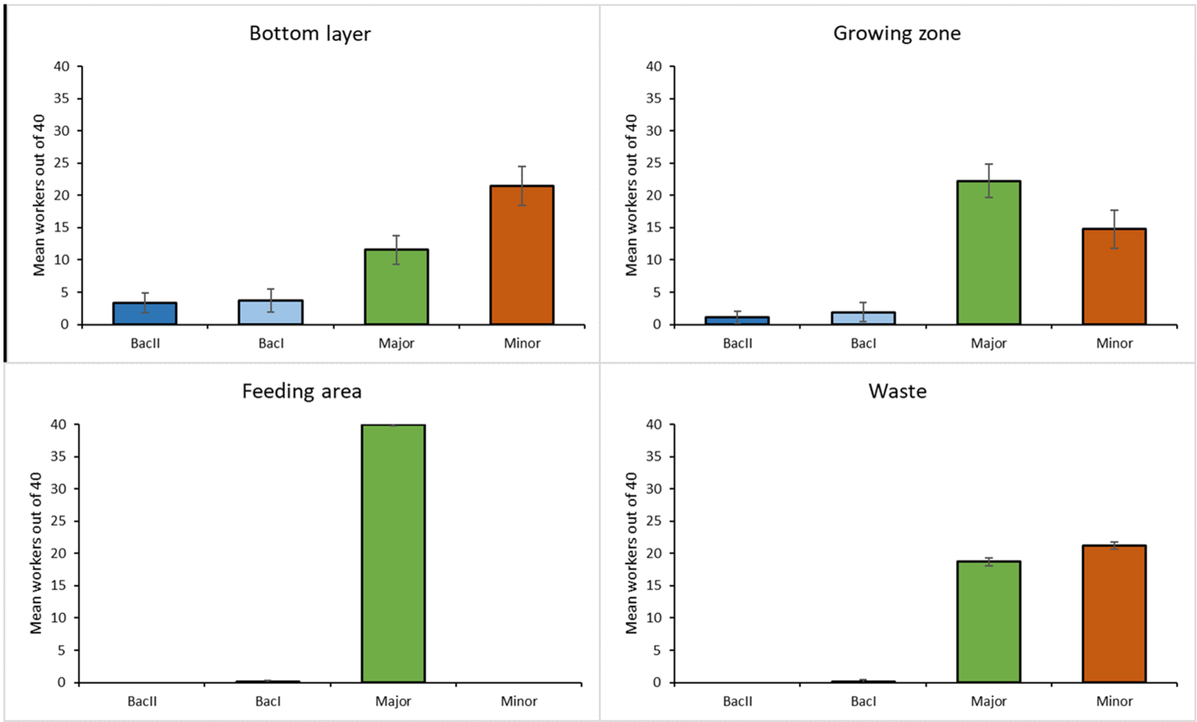

Fig. 1 The location preference of the workers covered with bacteria is shown. Each area was scanned 20 times. While there were more ants with external bacteria on the old fungi than on the growing zone, no such workers could be found in the feeding area or on the waste

$p < 0.05$) while differences between colonies were not significant. An overview is given in Fig. 2. Our investigations indicate that major workers without external bacterial layer are significantly (FET, $p < 0.05$) associated with behaviors involving the treatment of leaves, such as holding, licking, scraping, puncturing

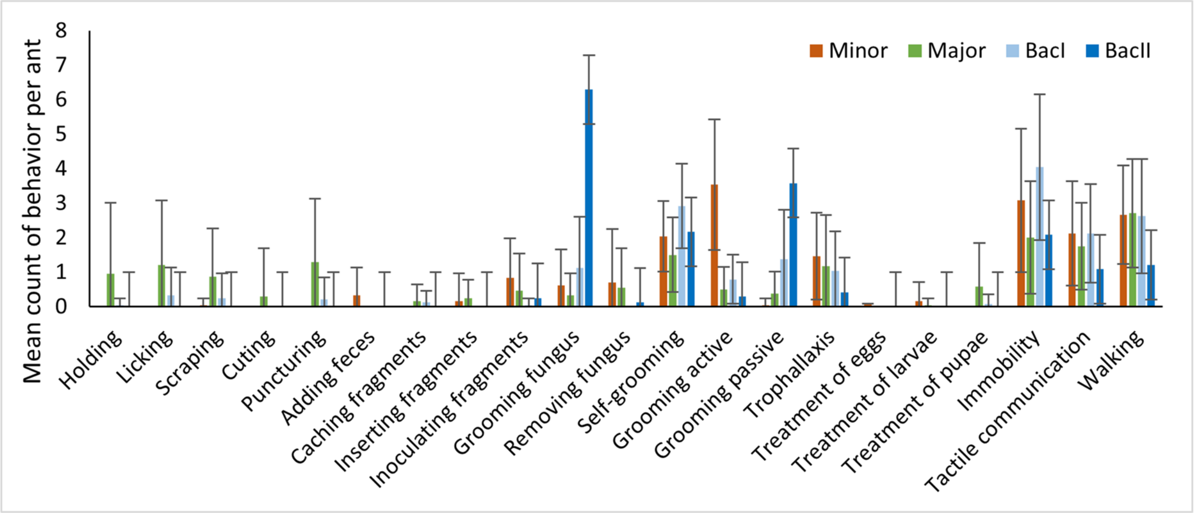

Fig. 2 Bar charts showing the behavioral patterns of the three colonies of *A. octospinosus* under observation

and cutting. The treatment of eggs only occurred in minor workers (FET, $p < 0.05$). Most interestingly, the BacII workers performed significantly more fungus grooming (FET, $p < 0.05$) and passive grooming behavior (FET, $p < 0.05$) than all the other types of workers. While BacII performed 6.3 ± 1.9 fungus grooming and 3.6 ± 1.7 passive grooming behavior, BacI performed 1.1 ± 1.5 and 1.4 ± 1.4, major performed 0.3 ± 0.6 and 0.4 ± 0.6 and minor performed 0.6 ± 1.0 and 0.0 ± 0.2 of fungus grooming and passive grooming behavior. Thus, they differ a lot from the workers of the major type, as BacII perform nearly no treatment of leaves while major workers demonstrate nearly no fungus grooming behavior. Behavioral pattern with significant differences between worker groups are highlighted in Fig. 3.

Discussion

The results from experiments focusing on the ants´ location preference suggest that the existence of external symbiotic microbiota on the ants is associated with the workers' location. A link between cuticular microbiota and the workers' preference of location might be a specific function of the microbiota. Possible functions depending on the location, especially the nest, would be the treatment of the fungi or the brood (Poulsen et al. 2002; Ivens et al. 2009). In line with previous research, our study showed that ants covered with external microbiota (BacI and BacII) have a high preference in location for the bottom of the nest where the old fungus accumulate (Poulsen et al. 2002). This is likely because the oldest fungus for location at the bottom of the nest can be infected by pathogens much more easily than the young fungus growing on the top (Currie 2001a), as on the top of the nest all of the new plant material is groomed by the ants and treated in the infrabuccal pocket (Quinlan and Cherrett 1978; Little et al. 2003). As a result, workers with a cuticular microbiome with a specific anti-pathogen function will only be effective if they are located in the nest, especially on the bottom of the nest, and not outside. This is confirmed by our results, as BacII and BacI only occur in the nest, with a significant preference for the old fungus.

The behavioral observations indicate a significant association between BacII and fungus grooming and passive grooming behavior, when compared with major, minor and BacI. This is surprising as Abramowski et al. (2011), who did not distinguish between different types of major workers, showed that minor workers are the caste performing the most grooming behavior. Thus, BacII behaves in a very specific way and is much more similar to minor workers than to major workers in this respect. This

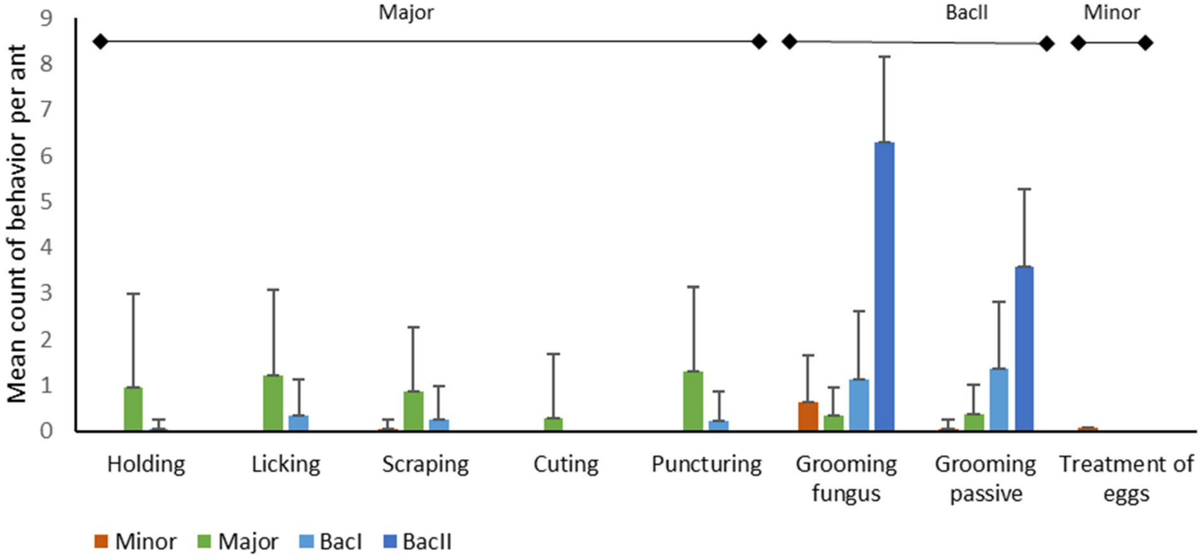

Fig. 3 Bar charts highlight behavioral differences between types of workers. Lines point out behaviors associated with a specific worker type. BacII shows much more fungus grooming and passive grooming behavior than BacI, major and minor

difference in behavioral pattern suggests that BacII have a sanitary function, as the grooming of fungus by a worker with a high amount of antibiotic-producing microbiota is an effective way to inhibit pathogens (Poulsen et al. 2003; Ugelvig and Cremer 2007; Walker and Hughes 2009; Abramowski et al. 2011; Ishak et al. 2011). The passive grooming of these workers can improve the spread of the symbiotic bacteria within the colony (Poulsen et al. 2003). This would enhance the effectiveness of BacII's treatment of parasitic fungi. Both grooming behavior are reported as behavioral defense against pathogens (Nilsson-Møller et al. 2018). Interestingly, the presence of *Escovopsis* has not been proven in our study which might explain the differences to the results Abramowski. Therefore, BacII might serve as unspecific defense strategy. In further research, it should be investigated if the presence of *Escovopsi*s leads to an up-regulation of BacII.

Overall, our results indicate that workers completely covered with *Pseudonocardia* have a specialized role. This is confirmed by the work of (Marsh et al. 2014), which showed that there is evidence that after workers have eclosed, there is a sensitive phase of two days which determines whether they will be covered with bacteria later if they are exposed to such bacteria. A possible mechanism therefore might be the adaption of elaborate cuticular crypts, in which the bacteria can be reared. These structures have been highly modified across the fungus-growing ants' evolutionary history (Currie et al. 2006). Our findings, combined with previous research showing that bacterial growth can be induced by the presence of Escovopsis (Currie et al. 2003), support the hypothesis that these workers play a sanitary role in the ant-fungus-symbiosis (Ratnieks and Anderson 1999; Beshers and Fewell 2001; Hart et al. 2002; Poulsen et al. 2003; Ugelvig and Cremer 2007; Ivens et al. 2009; Walker and Hughes 2009; Abramowski et al. 2011). Behavioral specialization leading to a sanitary function would be very adaptive in the arms race between leafcutter ants, their symbionts and pathogens (Pathak et al. 2019). Furthermore, such a function would make those specialized workers much more interesting for further research, such as pharmaceutical research, as it could open up opportunities to find as yet unknown antimicrobials (Currie et al. 2003; Seipke et al. 2011; Holmes et al. 2016). Additionally, further studies should be conducted to prove whether workers with a symbiotic bacterial layer can be described as a separate caste, as at every age most of the majors and minors have no bacteria or a reduced bacterial layer on their propleural plate (Andersen et al. 2015), or if the change in the external bacterial layer during the ants' life history is responsible for the specialized role of these workers.

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Author Contribution JPF conceived the research. JPF and FB designed the experiments on location preference and behavioral pattern. All authors read and approved the manuscript.

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Declarations

Conflict of Interest The authors declare no conflict of interest.

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