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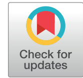

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ABSTRACT

The ant *Lasius fuliginosus* stabilizes its carton-nest structures through the growth of fungi. Here we investigated the fungal and bacterial community composition in nest-carton and adjoining soil of *L. fuliginosus*. We found that fungal communities in the nest were stable and distinct from surrounding soil over 2 y, while bacterial communities were not stable with the differentiation between nest and surrounding soil changing over the years. This suggests that in contrast to bacterial communities, fungal communities in the nest are actively managed by the ant *L. fuliginosus*, a result that was corroborated by additional growth assays. In these assays we found that an antagonistic fungus was inhibited when incubated with extracts of ant body parts, while fungal associates were not only not affected but even partly favoured in their growth. Overall our results support a role of host secretions in shaping the association between *L. fuliginosus* and fungal partners.

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1. Introduction

Animals commonly host a wide variety of microbes and closely interact with microbes in their environment. Ants are no exception to this and are known to entertain diverse associations with microbial partners (Zientz et al., 2005; Russell et al., 2017). These ant-microbe associations can show a continuum of specificity ranging from highly variable (Vasse et al., 2017) to highly specialized with exclusive vertical transfer (Sauer et al., 2000) and can comprise bacterial as well as fungal species that potentially interact in complex networks (Little and Currie, 2008). Among these associations the cultivation of fungi either as a food source or for structural support of the nest has received considerable attention (Chomicki and Renner, 2017), especially in the well-studied case of fungus-growing ants that depend on the cultivation of basidiomycete fungi as a food source (Mueller et al., 1998; Munkacsi et al., 2004; Kooij et al., 2015). Fungal cultivation is also found in ants that nest in specialized hollow plant structures, termed domatia, and in ants that fabricate carton, a cardboard-like material from masticated plant fibres, for the construction of nests. Both of these forms of ant-fungus association are widespread and involve a range of unrelated ant species (Maschwitz and Hölldobler, 1970; Kaufmann and Maschwitz, 2006; Leroy et al., 2011; Mayer et al., 2014). Numerous recent investigations have started to characterize these associations and to identify and describe the involved fungal

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partners. These studies revealed that fungal partners inside domatia and carton-nests mostly belong to the order Chaetothyriales in the Ascomycota, and, to a lesser extent, Capnodiales and the family Venturiaceae (Schlick-Steiner et al., 2008; Defossez et al., 2009; Mayer and Voglmayr, 2009; Ruiz-González et al., 2011; Voglmayr et al., 2011; Nepel et al., 2014; Kokolo et al., 2016; Nepel et al., 2016; but see Baker et al., 2017 for the presence of other fungi). Many of these studies used culture-dependent approaches to detect fungal associates, which are potentially limited in uncovering fungal associates and the fungal community composition in the nest. In addition, the fungal and bacterial community found in the environment of these ant-nests, e.g. the nest refuse pile or foraging ground, has so far received little attention (but see Baker et al., 2017 and Lucas et al., 2017 for a comparative community analysis of the nest versus the environment).

Inclusion of all microbes in studies of ant-fungus associations is however important, as host-microbe associations are known to be shaped not only by the abiotic environment (Russell and Moran, 2006; Colman et al., 2012) but also by the biotic environment, i.e. by horizontal transfer with free-living microbial populations (Adair and Douglas, 2017), and by microbe-microbe interactions (Goto et al., 2006; Dittmer et al., 2016). Potential environmental acquisition and interactions with adjoining microbial communities may play an important role in shaping microbial communities in ants building carton-structures. In carton-structures, several fungi appear to colonise the carton and to show a low specificity with regard to the identity of carton-making ant (Mayer and Voglmayr, 2009; Nepel et al., 2014, but see Schlick-Steiner et al., 2008; Ruiz-González et al., 2011 for the presence of only few or a single dominant fungus). In line with this, a recent phylogeny of chaetothyrialean fungi with representative samples from ant-fungus associations, but also free-living species, showed that ant-associated fungal taxa are not necessarily host-specific, but can occur in niches other than ant nests (Vasse et al., 2017).

Another important factor shaping host-microbe associations are the hosts themselves (Duarte et al., 2017). To maintain stable associations with microbial partners, organisms need to tend associates and to manage them, i.e. to control and manipulate them. Management of the microbial community surrounding an organism via externalised antimicrobial substances - a form of external immune defence - is known from several different animals (Otti et al., 2014) and has likely been favoured in social insects due to their lifestyle (Tragust, 2016). In ants that cultivate fungi in domatia and carton-nests, evidence for the active management of fungal associates comes so far only from the behavioural observation that ants apparently masticate and groom their fungal associates and that in the absence of ants the fungus starts to show uncontrolled growth (Maschwitz and Hölldobler, 1970; Dejean et al., 2005; Defossez et al., 2009; Mayer and Voglmayr, 2009; Ruiz-González et al., 2011). Active management of fungi could however also be achieved through chemical control, either with the help of microbial symbionts that produce bioactive compounds, as seen in many Hymenoptera (see Kaltenpoth and Engl, 2014 for a review of defensive microbial symbionts), or through the use of antimicrobial secretions from exocrine glands that are located all over the body of most ants (Morgan, 2008; Vander Meer, 2012; Tragust, 2016).

Here we aim to elucidate patterns in the metacommunity composition of carton-nest material and adjoining soil over time as well as the active management of fungal associates via chemical substances in the temperate ant *Lasius fuliginosus*. This ant represents one of the first recorded examples of fungus cultivation apart from fungus-growing ants. In this association ants nourish fungi with honeydew to bind shredded wood or soil into a cardboard-like building material (Maschwitz and Hölldobler, 1970). Nest chambers providing shelter for the queen, brood, and workers are constructed from this material. Carton-nests are built along tree roots underground or in hollow trees close to the ground. Morphological and molecular characterization of fungal isolates from the carton-nest of several temperate *Lasius* species including *L. fuliginosus* revealed a complex association of several species of fungi with the different ant species (Schlick-Steiner et al., 2008). In a first step of the present study, we investigated the composition of the whole metacommunity including bacteria and fungi, not only in the carton-structure but also the adjoining soil at two sampling time points via the fingerprinting method ARISA (Automated Ribosomal Intergenic Spacer Analysis). This allowed us to investigate whether the same or different processes govern the diversity and composition of fungi and bacteria in the nest-carton and adjoining soil over time. In accordance with *L. fuliginosus* cultivating fungal associates in their carton-nest, we expected to find a higher stability and similarity of fungal communities in the nest-carton compared to adjoining soil, while bacterial communities were not necessarily expected to follow this pattern. In a second step, we aimed to reveal whether *L. fuliginosus* ants have the potential to manage the fungal community in their carton-nests via chemical control. In growth assays we incubated supernatants from body parts that bear antimicrobial substance producing exocrine glands with fungal solutions that were either derived from the carton-nest or from an antagonist of the ants, an entomopathogenic fungus. We expected an inhibitory effect against the entomopathogenic fungus, while fungi from the carton-nest were expected not to be affected.

2. Materials and methods

2.1. Soil and nest material collection

Nest material and adjoining soil of nine different *L. fuliginosus* colonies were collected from nine different locations during August 2013 near Bayreuth (Germany, Bavaria, see Supplementary Table S1). The same locations were revisited in June 2014 and nest material and adjoining soil re-collected. Sampling was repeated to investigate the stability of microbial community composition over time, as this provides insight into whether associations are actively managed and maintained. Nest material was collected manually (using gloves sterilised with ethanol) by grabbing into the nest entrance and recovering nest material in a depth of 40–70 cm belowground. Soil samples were taken adjacent to the nest entrance, at approximately the same depth as the nest material with an ethanol cleaned shovel. Ant workers, brood as well as bigger debris such as leaves, and stones were removed from the nest and soil material, which subsequently was transferred into sterile tubes (50 ml; Greiner) and stored at −20 °C within 3 h after collection until DNA extraction.

2.2. Microbial community analyses: DNA-Extraction, ITS-Amplification and ARISA capillary electrophoresis

Microbial community composition was characterized by automated ribosomal intergenic spacer analysis (ARISA), a length heterogeneity polymerase chain reaction (PCR)-based approach to rapidly generate whole-community “fingerprints” of bacterial and fungal assemblages (Fisher and Triplett, 1999; Ranjard et al., 2001). Community fingerprinting methods are limited in their capacity to capture the presence of rare taxa and derived estimates of the abundance of taxa might be inaccurate (Bent et al., 2007; Bent and Forney, 2008). Nowadays they are mostly supplanted by higher resolution methods, i.e. high-throughput sequencing. Despite their limitations, community fingerprinting methods still represent a cost-effective and sensitive method that can perform just as well as higher resolution methods when investigating ecological patterns

in microbial communities at multiple scales (van Dorst et al., 2014; Powell et al., 2015). ARISA uses universal primers to amplify the ITS region of bacterial ribosomal RNA operons or the equivalent ITS1-5.8S-ITS2 region in fungi. Because these hypervariable stretches of DNA vary in length across taxonomic groups (on strain, species, or genus level), the collection of ARISA amplicons represents the taxonomic diversity and composition of that sample. However, as the taxonomic resolution of ARISA does not correspond to the Linnaean binomial nomenclature and does not rest on phylogenetic relationships, we refrain from talking about microbial "species" and hereafter refer to these ARISA-defined groups as ARISA-types. Although ARISA and other fingerprinting methods are suitable to investigate microbial community patterns, these methods hinder a direct comparison with a previous study in the same system (Schlick-Steiner et al., 2008) as comparable taxonomic information is missing. Moreover, the methodological approaches of our and the previous study, both introduce potential biases to the estimation of microbial diversity (over- and underestimation, respectively) and thus a direct comparison of the results is seriously hampered.

To characterize the microbial community composition, we extracted genomic DNA from nest and soil material with the MN NucleoSpin®Soil Kit (Macherey-Nagel) according to the manufacturer's protocol. The horizontal vortex time was expanded to 12 min to get a homogeneously mixed sample. Extraction was carried out on 250–500 mg material. Lysis Buffer SL1 was used for the extraction and the extracted DNA resolved in 30 μl Buffer SE and stored at −20 °C.

After DNA extraction, ITS fragments were amplified from genomic DNA (about 5 ng) in a 12.5 μl reaction volume consisting of 1.5 mM $MgCl_2$, 1 × PCR buffer (Promega GoTaq Kit), 0.2 mM dNTP Mix, 0.32 U GoTaq Hot Start DNA polymerase (www.promega.de) and 0.5 μM of each primer using the following PCR settings: initial activation of the hot-start polymerase at 95 °C for 2 min, followed by 35 cycles at 94 °C for 30 s, 56 °C (fungi, fARISA) or 55 °C (bacteria, bARISA) for 30 s, 72 °C for 60 s, followed by a final extension at 72 °C for 5 min. For the fARISA primer ITS1F-Z (Weig et al., 2013) and ITS2 (White et al., 1990) were used, while for the bARISA ITSF and ITS-Reub (Cardinale et al., 2004) were used. Primers ITS1F-Z and ITSF were labelled with fluorescent dyes BMN-5 and BMN-6, respectively (biomers.net GmbH, Ulm, Germany).

PCR-amplified ITS fragments of the bARISA and fARISA were separated on a GenomeLab GeXP Genetic Analysis System (AB Sciex) and the electropherogram data were analysed with GenomeLab GeXP Software (ver. 10.2). Peaks below the threshold of 2% total peak intensity were ignored. The bin-width was set to 2 nucleotides using an AFLP-type size calling approach, to correct for minor differences in migration time observed for larger, slower-migrating fragments, which resulted in a primary data matrix (sample by fragment size) of absolute peak heights.

2.3. Fungal growth assays

To investigate the potential management of fungal associates by the ants we used a growth assay to determine any inhibitory or growth promoting activity of ant tissue supernatant compared to controls without ant tissue supernatant. Growth assays were performed with three different fungus solutions: (1) nest-carton material (laboratory colony of *L. fuliginosus* that was collected in 2013, location ÖBG1, Supplementary Table S1), (2) a fungus from the order Capnodiales (donated by Birgit Schlick-Steiner and Florian Steiner and originally isolated from nest-carton material of *L. fuliginosus* in Austria, see Schlick-Steiner et al., 2008 and Supplementary Material for identification, GenBank Acc. No. MH629975) and (3) isolates of the entomopathogenic fungus *Metarhizium* sp. (two isolates: strain KVL 03–143, Ma275 obtained from the Faculty of Life Sciences, University of Copenhagen, Denmark, originally isolated from *Cydia pomonella*, Lepidoptera, Torticidae, in Germany, see Hughes et al., 2004a, and strain Ma97, originally isolated from unknown Lepidoptera in the Philippines, see Stephan and Zimmermann, 1998). *Metarhizium* fungi frequently occur in soil and are responsible for natural infections of ants (Hughes et al., 2004b; Reber and Chapuisat, 2012). As an inhibitory activity of exocrine gland substances against *Metarhizium* sp. has been shown in several ant species (Morgan, 2008; Yek and Mueller, 2011; Vander Meer, 2012; Tragust et al., 2013), these fungi served as a positive control for growth inhibition. The fungus of the order Capnodiales instead, served as a positive control for growth effects of the fungus solution derived from the nest-carton material, as it was previously identified as a fungal associate of *L. fuliginosus* (Schlick-Steiner et al., 2008). Both, strains of *Metarhizium* sp. and the fungus from the order Capnodiales, were chosen to be of foreign geographic origin to mitigate possible effects of local adaptation between ants and fungal associates in the fungal solution derived from nest-carton material (Kraemer and Boynton, 2017). 350 mg of nest-carton material and of the capnodialean fungus were weighted and homogenized in 1 ml sterile water. *Metarhizium* sp. fungi were cultured as described in Konrad et al. (2015) and a working concentration of 1×10^8 in 0.01% Triton X-100 was used. Each of these fungal solutions was subsequently tested against the supernatant of whole ant tissue from different body parts (head, thorax and gaster). Prior to dissection, ants were cold anaesthetized for 15 min on ice. To equalize weight between ant body parts, four, six and two body parts were pooled for each of the head, thorax and gaster ant body part samples, respectively. In addition, each body part sample was weighed. Body part samples were put in reaction tubes, 50 μl of sterile, bi-distilled water was added, and the body parts were mechanically crushed with a sterile pestle. Before used in the growth assay each body part sample was vortexed and centrifuged for 5 min at 6000 × *g* and only the supernatant was used. Ants were taken from the same laboratory colony from which the fungal solution of the nest-carton material was derived. We did not include a variety of biological replicates of the fungal solutions and of ant body part supernatants from different *L. fuliginosus* colonies, as fungal growth assays served as a proof of principle experiment for the management of fungal associates via ant derived substances.

Growth assays followed the protocol of Konrad et al. (2012) with some modifications. Each body part sample was incubated with each fungus solution in 96-well microplates with Malt Extract Broth (15 g malt extract and 2.5 g peptone per 1000 ml water together with 0.1% streptomycin sulfate to inhibit unwanted bacterial growth) as a growth substrate. Each well in the microplates contained 130 μl Malt Extract Broth, 15 μl fungus solution and 5 μl ant body part supernatant. Pure fungus solutions without an ant body part sample served as baseline fungal growth controls (135 μl Malt Extract Broth and 15 μl fungus solution per well). Growth was measured as an increase in absorbance at 595 nm in a spectrophotometer (Synergy HT, BioTek) over the course of 18:30 h every 30 min at 23 °C.

Absorbance of all fungal solutions incubated with the different ant body part samples as well as the pure fungus solutions was measured simultaneously in one microplate. This allowed measurement of 8 replicates per combination of fungal solution and ant body part sample as well as pure fungus growth on one microplate. An additional growth measurement was performed on the next day with the same fungus solutions (stored at 4 °C overnight) but with freshly prepared ant body part samples, raising the number of replicates to 16. A few weeks later, two more growth measurements in another two more microplates were carried out in the same fashion with freshly prepared fungus solutions but exchanging the

Metarhizum sp. isolate and measuring only 7 replicates of pure fungus growth.

2.4. Statistical analyses

All statistical analyses were performed in R 3.4.3 (R Core Team, 2017). ARISA-type diversity of fungal and bacterial communities, in terms of observed number of ARISA types (ARISA type richness), was analysed using separate Linear Mixed Effects Models (LMM, package "lme4", Bates et al., 2015) that included sampling time (August 2013 or June 2014), sample type (nest or soil) as well as their interaction as predictors. The intercept and slope of sample location with respect to sampling time was included as a random effect in these models to account for the fact that soil and nest samples were repeatedly collected from the same location in two years. Statistical significance of predictors was assessed by Likelihood-Ratio-Tests (LR) on models with and without each predictor. PCR-based fingerprinting methods potentially yield a biased representation of the community composition due to preferential and nonspecific amplifications and/or heterogeneity in ribosomal DNA within or between species (Rajendhran and Gunasekaran, 2011) which will likely translate into biased diversity estimates (Crosby and Criddle, 2003; Bent and Forney, 2008). Despite these potential biases, we decided to analyse ARISA type richness, as we were more interested in richness patterns between sample types and over sampling time than in the absolute richness values. Also, inter-sample variation should not be affected by biased conditions because they similarly apply to all ARISA types and samples (Blackwood et al., 2003; Schütte et al., 2008).

Given that estimates of the abundance of taxa derived from community fingerprinting methods might be inaccurate (Bent et al., 2007; Bent and Forney, 2008), we converted the absolute peak heights of ARISA profiles into an absence/presence matrix of ARISA fragments before analysing differences in fungal and bacterial community composition between the collected samples based on the Jaccard dissimilarity index (function "distance", package "phyloseq", McMurdie and Holmes, 2013). Analyses were carried out following the guidelines for multivariate statistical techniques in microbial ecology of Ramette (2007) and Paliy and Shankar (2016) using exploratory (unconstrained) and interpretative (constrained) ordination methods in parallel. These ordination methods differ in that unconstrainted methods extract the highest amount of variance from the data alone and represent it on a few axes in multivariate space, while constrainted methods only represent and analyse variation that can be explained by provided variables. Nonmetric multidimensional scaling (NMDS) was used as unconstrained ordination method and analysis of principal coordinates (CAP) as constrained ordination method. Both methods allow the use of a distance matrix as data input and base the assumed relationship among variables on the chosen distance matrix. Differences in fungal and bacterial community profiles between sample type (nest or soil), sampling time (August 2013 or June 2014) and sampling location were visualized via ggplot2 (function "ggplot", package "ggplot2", Wickham, 2009) by first carrying out both ordination methods via the function "plot_ordination" (package "phyloseq", McMurdie and Holmes, 2013) and then passing the ordination results to ggplot2 for a custom plot. For CAP a model formula had to be provided (see below). In addition to representing relationships in an ordination plot we also tested for significant differences in fungal and bacterial community profiles via permutation tests. Two different permutation tests (999 runs) implemented in package "vegan" were performed via the function "adonis" (permutational multivariate analysis of variance using distance matrices) and via the function "anova.cca" (Permutation Test for Constrained Correspondence Analysis, Redundancy Analysis and Constrained Analysis of Principal Coordinates) (Oksanen et al., 2017). For both tests sample type (nest or soil), sampling time (August 2013 or June 2014) and their interaction were included as factors. For "adonis" we also included sample location as a factor to investigate whether communities differ between sampling locations and we restricted permutations within sample location (argument "strata" in call function "adonis"). The latter accounts for the fact that there is temporal and spatial correlation between samples from the same location and that data thus do not fulfil the assumption of exchangeability (Anderson, 2001). For "anova.cca" we included sample location as a conditioned variable (term "Condition" in call function "anova.cca"), which allows the removal of variation from a chosen variable from the analysis and thus a view on the effect of sampling time and sample type on differences in fungal and bacterial community profiles without the influence of sample location.

For statistical analysis of the growth assay we converted the raw absorbance values into relative growth values by dividing absorbance values obtained at the end of the growth assay (18:30 h) with values obtained at the beginning of the growth assay (0 h) for each replicate. Relative growth values were then analysed using separate Linear Mixed Effects Models (LMM, package "lme4", Bates et al., 2015) for each fungus solution, including the type of ant body part sample (head, thorax, gaster or none) as a predictor. Although we tried to equalize weight between body part samples by pooling four heads, six thoraxes and two gasters per body part sample, we also included weight as a predictor in each analysis. In addition, we added the day measurements were performed as a random effect to account for the fact that we used the same fungal solution for growth assays on two consecutive days as well as week to account for the fact that growth measurements were performed in separate weeks or in the case of *Metarhizium* sp. with another strain. Statistical significance of predictors was again assessed by Likelihood-Ratio-Test (LR) on models with and without each predictor. Pairwise comparisons between factor levels of a significant predictor were performed using Tukey post-hoc tests adjusting the family-wise error rate according to the method of Westfall (package "multcomp"; Bretz et al., 2011). In the model with the capnodialean fungus, we square root transformed the relative growth values to normalize the data.

3. Results

3.1. Fungal and bacterial community structure

The number of observed fungal ARISA types was constantly low in samples from the carton-nest of ants in August 2013 and June 2014 (mean ± s. e.: 19.67 ± 3.34 and 16.22 ± 2.01, respectively), while samples from soil next to ant nests showed higher numbers of observed fungal ARISA types and a change between sampling time points (25.78 ± 5.35 and 51.11 ± 6.95 for August 2013 and June 2014, respectively) (Fig. 1A; Table S2; LMM, LR-Test: sample type, i.e. nest or soil: $\chi^2 = 13.387$, $P < 0.001$; sampling time, i.e. August 2013 or June 2014: $\chi^2 = 3.389$, $P = 0.067$; interaction between sample type and sampling time: $\chi^2 = 10.276$, $P = 0.001$).

In contrast, the number of observed bacterial ARISA types was high in the nest and in the soil with a drop in numbers in nest samples from June 2014 compared to nest samples from August 2013 (nest: 119.78 ± 13.15 and 64.33 ± 13.05, soil: 120.00 ± 12.79 and 105.67 ± 19.26, for August 2013 and June 2014, respectively) which did however not result in a statistically significant interaction between sample type and sampling time (Fig. 1B; Table S2; LMM, LR-Test: sample type: $\chi^2 = 2.554$, $P = 0.110$; sampling time: $\chi^2 = 3.098$, $P = 0.078$; interaction between sample type and sampling time: $\chi^2 = 2.795$, $P = 0.095$).

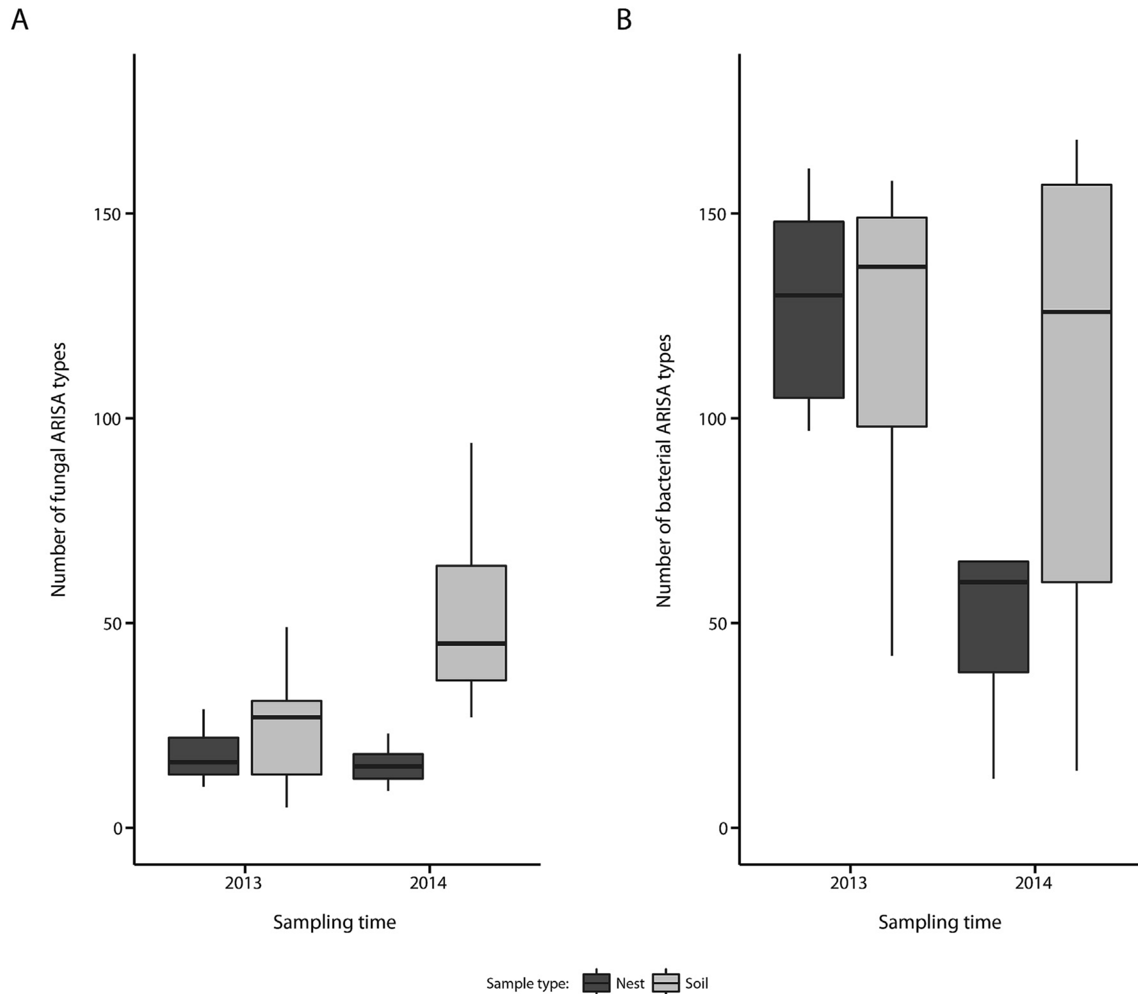

Fig. 1. Number of fungal (A) and bacterial ARISA types (B) in samples of the carton-nest (dark grey) and in samples of soil surrounding the nest (light grey) of the ant *Lasius fuliginosus* taken in August 2013 and in June 2014 (N = 9 for each sample type and sample time). While the observed number of fungal ARISA types was low and stable in the nest compared to higher and variable numbers in the soil (LMM, LR-Test: interaction between sample type and sampling time: $\chi^2 = 10.276$, $P = 0.001$), bacterial ARISA types, albeit more variable in the nest over time, were statistically not different between nest and soil for both years (LMM, LR-Test: sample type: $\chi^2 = 2.554$, $P = 0.110$; sampling time: $\chi^2 = 3.098$, $P = 0.078$; interaction between sample type and sampling time: $\chi^2 = 2.795$, $P = 0.095$). Boxplots show the median, as well as the upper and lower quartiles. The whiskers encompass 1.5 times the interquartile range.

Similar to ARISA-type diversity, distinct patterns of fungal and bacterial community composition differences were obtained when we analysed variation in fungal and bacterial community composition within and among sample type, sampling time and sample locations. Both, fungal and bacterial community composition varied significantly among sample locations, and to a lesser extent sample type and sampling time (adonis; fungal community composition: sample location: $pseudo\text{-}F_{1,24} = 1.452$, $R^2 = 0.274$, $P = 0.001$, sample type: $pseudo\text{-}F_{1,24} = 3.768$, $R^2 = 0.089$, $P = 0.001$, sampling time: $pseudo\text{-}F_{1,24} = 1.364$, $R^2 = 0.032$, $P = 0.071$, interaction between sampling time and sample type: $pseudo\text{-}F_{1,24} = 1.618$, $R^2 = 0.038$, $P = 0.024$; bacterial community composition: sample location: $pseudo\text{-}F_{1,24} = 1.241$ $R^2 = 0.256$, $P = 0.001$, sample type: $pseudo\text{-}F_{1,24} = 1.358$, $R^2 = 0.035$, $P = 0.046$, sampling time: $pseudo\text{-}F_{1,24} = 2.186$, $R^2 = 0.056$, $P = 0.001$, interaction between sampling time and sample type: $pseudo\text{-}F_{1,24} = 1.325$, $R^2 = 0.034$, $P = 0.062$). Visualisation of the fungal community composition via the unconstrained ordination NMDS revealed that samples from the nest were similar for both sampling time points, while samples from the soil formed distinct groups for each year (Fig. 2A). For the bacterial community, samples from the nest-carton and soil in June 2014 formed somewhat distinct groups in the NMDS plot, while samples from August 2013 grouped closely together (Fig. 2B). The influence of sample type and sampling time on community composition after accounting for variation caused by sample location confirmed this pattern in the constrained ordination. Visualisation of fungal community composition via CAP now clearly revealed that nest samples from both years were similar to each other, while samples from the soil each formed a separate group per year (Fig. 2C; anova.cca: sample type: $pseudo\text{-}F_{1,24} = 3.768$, $P = 0.001$, sampling time: $pseudo\text{-}F_{1,24} = 1.364$, $P = 0.091$, interaction between sampling time and sample type: $pseudo\text{-}F_{1,24} = 1.618$, $P = 0.027$). Community composition of bacteria in contrast, showed a close grouping of nest and soil samples for August 2013, while nest and soil samples from June 2014 were separated from August 2013 samples and from each other (Fig. 2D; anova.cca: sample type: $pseudo\text{-}F_{1,24} = 1.358$, $P = 0.065$, sampling time: $pseudo\text{-}F_{1,24} = 2.186$, $P = 0.002$, interaction between sampling time and sample type: $pseudo\text{-}F_{1,24} = 1.325$, $P = 0.075$).

3.2. Fungal growth assays

The addition of ant body part supernatant had a significant influence on the growth of all three fungal solutions tested (LMM; LR-

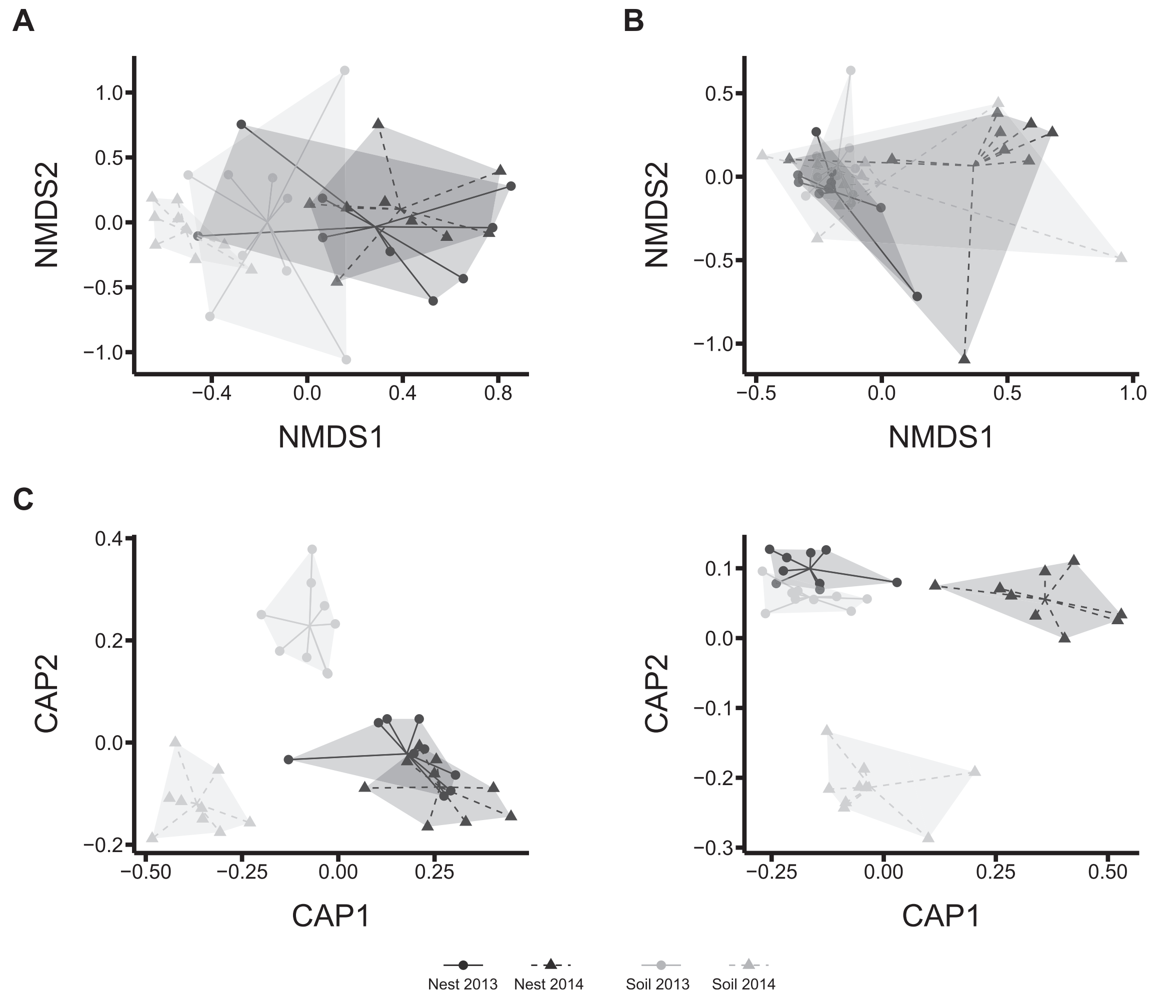

Fig. 2. Differences in fungal (A,C) and bacterial (B,D) community composition between samples of the carton-nest (dark grey symbols) and samples of soil adjoining the nest (light grey symbols) of the ant *Lasius fuliginosus* taken in August 2013 (dots) and in June 2014 (triangles). Shaded areas (light grey: soil, dark grey: nest) enclose all sample points in a group, while lines (solid: August 2013, dashed: June 2014) connect samples to the respective group centroids. Both, the unconstrained non-metric multidimensional scaling, NMDS (A,B) and the constrained analysis of principal coordinates, CAP (C,D) were based on the Jaccard dissimilarity index calculated from an absence/presence matrix of microbial ARISA-types. For the latter, variation from the different sampling locations was factored out. Fungal communities (A,C) were similar between nest-carton from both years but distinct from the soil, while bacterial communities (B,D) were similar between nest-carton and soil in August 2013 but formed distinct groups in June 2014. NMDS-2d stress values are 0.223 and 0.175 for fungi and bacteria, respectively.

Test; *Metarhizium* sp.: $\chi^2 = 17.147$, $P < 0.001$; capnodialean fungus: $\chi^2 = 63.408$, $P < 0.001$; nest-carton material: $\chi^2 = 21.453$, $P < 0.001$). While ant body part samples significantly inhibited the growth of the entomopathogenic fungus *Metarhizium* sp. compared to pure fungus growth (Fig. 3A; post-hoc Tukey comparisons; pure fungus versus ant body part sample: all comparisons: $P < 0.01$, except gaster versus thorax: $P = 0.455$), both the growth of the capnodialean fungus (Fig. 3B) as well as the fungi in a solution derived from nest-carton material (Fig. 3C) were not affected when incubated with supernatant from the head and were promoted compared to pure fungus growth when incubated with ant body part supernatant of the thorax and gaster (post-hoc Tukey comparisons; capnodialean fungus: all comparisons $P < 0.05$ except head vs. pure fungus: $P = 0.065$; fungus solution derived from nest-carton material: all comparisons $P < 0.05$, except head vs. pure fungus: $P = 0.373$). Weight of the ant body part sample did not influence growth of *Metharizium* sp. (LMM; LR-Test: $\chi^2 = 1.413$, $P = 0.235$) and the fungal solution derived from the nest-carton material (LMM; LR-Test: $\chi^2 = 0.732$, $P = 0.392$) but had a slight negative influence on the growth of the capnodialean fungus (LMM; LR-Test: $\chi^2 = 0.480$, $P = 0.028$).

4. Discussion

Our results on fungal communities of nest-carton from several colonies of the ant *L. fuliginosus* show that they are similar and stable over two sampling times, August 2013 and June 2014, but distinct from adjoining soil, containing a lower number of fungal ARISA types in June 2014 (Fig. 1A; Fig. 2A,C). In contrast, bacterial communities of nest-carton and adjoining soil grouped closely together in August 2013 but were distinct in June 2014 (Fig. 2B,D). The stability and similarity of fungal communities in the nest suggest that fungal communities are actively managed by the ant *L. fuliginosus*, a hypothesis supported by our growth assays. In these growth assays we found that an entomopathogenic fungus, *Metarhizium* sp., was inhibited when incubated with supernatants of ant body parts, while a previously identified fungal associate and the fungal material from the carton-nest were slightly favoured in their growth (Fig. 3).

Even though we found distinct fungal communities between nest-carton and adjoining soil, both fungal and bacterial communities were strongly influenced by sampling location and to a lesser extent by sampling time (Fig. 2). This is in accord with studies on

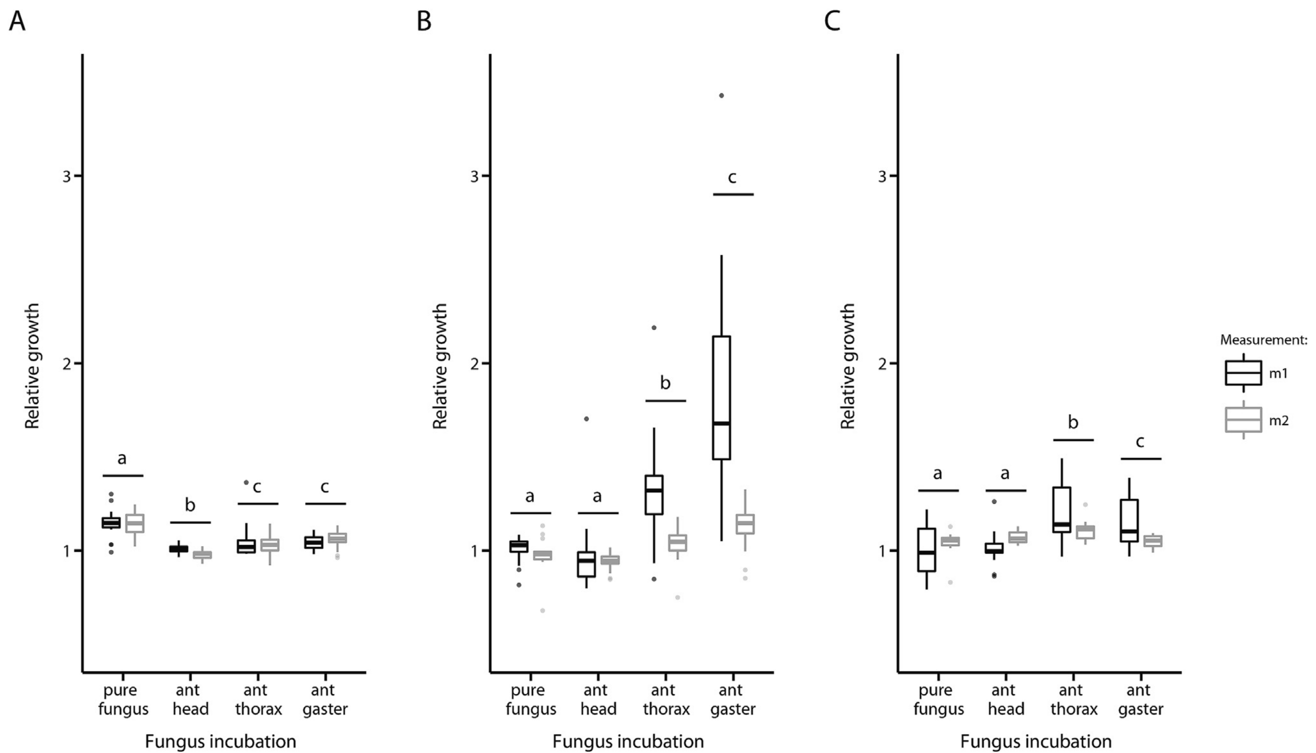

Fig. 3. Growth of antagonistic entomopathogenic fungi *Metarhizium* sp. (A), a capnodialean fungal associate (B), and a fungal solution derived from the carton-nest (C) either in pure culture (N = 30) or incubated with the supernatant of the body parts head, thorax or gaster of the ant *Lasius fuliginosus* (N = 32 each). Growth values lower than the growth values of the pure culture indicate growth inhibition by ant body parts and values greater than values of the pure culture indicate growth promotion. While the supernatant of the ant body parts inhibited the entomopathogenic fungi, it tended to promote the growth of the fungal solution derived from the nest material as well as the capnodialean fungal associate. Boxplots show the median relative growth, as well as the upper and lower quartiles. The whiskers encompass 1.5 times the interquartile range. Dots represent outliers. Data from two growth measurements performed at an interval of several weeks (included as a random effect in the statistical models but represented in the figure as separate boxplots to show the variability between measurements) are shown in black and grey, respectively (N = 16 for each growth measurement and ant body part sample; N = 15 for each growth measurement of fungal pure culture). In the case of *Metarhizium* sp. two different strains of the fungus were used for the two measurements. Small letters with horizontal black lines indicate statistically significant different groups at $\alpha < 0.05$.

fungal and bacterial communities of fungus-growing ants which also found that sampling time, as well as sample location had a strong influence on structuring the microbial communities in the fungus garden (Rodrigues et al., 2011; Kellner et al., 2015; Reis et al., 2015; Pereira et al., 2016). It was suggested that differences in microbial communities over time and space were due to changes in the plant substratum and its associated microbial communities foraged by fungus-growing ants and fed to the fungus garden (Rodrigues et al., 2011; Reis et al., 2015; Pereira et al., 2016). As *L. fuliginosus* ants use shredded plant material to construct their carton-nests and re-locate the nest and nest-carton depending on temperature and season (Maschwitz and Hölldobler, 1970) a similar influence of building material on microbial communities associated with the carton-nest might apply. The influence of sampling time and location on the microbial community composition in nest-carton of *L. fuliginosus*, irrespective of the underlying cause, emphasises the importance of considering the microbial meta-community of an organism, including communities in the environment, as microbial communities are connected through complex networks of transmission and interaction (Adair and Douglas, 2017; Foster et al., 2017).

In line with the hypothesis that ants influence microbial communities surrounding them (Otti et al., 2014; Tragust, 2016; Duarte et al., 2017), several authors report a microbial shift between nest soil and soil adjacent to the nest for several ant species, i.e. fire ants (Zettler et al., 2002; Ishak et al., 2011), harvester ants (Ginzburg et al., 2008), garden ants and common red ants (Dauber et al., 2001), wood ants (Duff et al., 2016) and fungus-growing ants (Ishak et al., 2011; Rodrigues et al., 2014; but see Kellner et al., 2015). For ants that engage in fungal cultivation in domatia plant structures or nest-carton the microbial metacommunity, including the community found in the environment of the nest of these ants, has so far received little attention. Bacterial communities in nest-carton of the ant *Azteca trigona* were not different from bacterial communities of adjoining soil (Lucas et al., 2017), a result paralleled in investigations of bacterial communities in the fungus garden of fungus-growing ants compared to adjoining soil (Kellner et al., 2015; but see Ishak et al., 2011 for a slight separation). In contrast, fungal communities in ant-inhabited domatia of the plant *Vachellia drepanolobium* were distinct from fungal communities found on leaves of the same plant (Baker et al., 2017). These studies concluded that the environment is the main source for bacterial colonisation of nest structures in *A. trigona* ants, while fungal communities in domatia of the ant-plant *V. drepanolobium* are shaped by the ant that inhabits them. Given our results, we similarly conclude that the ant *L. fuliginosus* shapes the fungal community in its carton-nest. Although we cannot rule out other factors that might have shaped the observed community patterns, e.g. microbial competition in the nest material, it is likely that *L. fuliginosus* either indirectly, through the creation of stable abiotic conditions, e.g. temperature and humidity, or directly, through chemical substances (see below), manages the fungal community in its nest. Recently, it has been proposed that animals in general might shape the microbial community surrounding them either through preservation, reducing the number of microbes, or through induction of microbial community shifts (Duarte et al., 2017). Our

results indicate that *L. fuliginosus* potentially preserves the fungal community associated with its nest-carton and reduces the growth of other, unwanted fungi, as the fungal community composition of the carton-nest was similar and stable over time (Fig. 2A,C), reduced in diversity of fungal ARISA-types compared to adjoining soil in June 2014 (Fig. 1A) and different in composition from adjoining soil (Fig. 2A,C). Microbial community patterns were different for bacteria (Fig. 1B; Fig. 2B,D), indicating that the observed patterns for the fungal community are not an artefact of some general microbial shift over time and that different processes influence bacterial and fungal communities.

The potential of *L. fuliginosus* to manage fungal associates in its carton-nest is indicated by the results of our growth assay. The growth of the entomopathogenic fungus *Metarhizium* sp. was inhibited when incubated with the supernatant of ant body parts, i.e. the head, thorax or gaster of *L. fuliginosus*, compared to the growth without the supernatant of ant body parts (Fig. 3A). In contrast, a capnodialean fungus previously identified as a fungal associate in the carton-nest of *L. fuliginosus* (Schlick-Steiner et al., 2008) as well as fungi in a solution derived from the carton-nest were not inhibited (Fig. 3B and C). Growth inhibition of *Metarhizium* sp. is likely to result from the antimicrobial activity of chemical substances from exocrine glands. The mandibular glands located in the head, the metapleural glands located in the thorax and the poison gland located in the gaster contain antimicrobial substances in a variety of ant species, some of them with an inhibitory effect against *Metarhizium* sp. (Morgan, 2008; Yek and Mueller, 2011; Vander Meer, 2012 and references therein). In *L. fuliginosus* the antimicrobial compound dendrolasin from the mandibular gland in the head (Pavan, 1956; Akino et al., 1995) and formic acid from the poison gland in the gaster (Tragust et al., 2013) could have caused the observed growth inhibition of *Metarhizium* sp. In addition, several potential bacterial symbionts that produce antifungal compounds have recently been described from the head of *L. fuliginosus* (Liu et al., 2016a, 2016b; Shen et al., 2017; Ye et al., 2017). The presence of bacterial symbionts with the ability to produce antifungal compounds has also been detected in other ants fabricating carton-nests and in ants inhabiting domatia (Seipke et al., 2012, 2013; Gao et al., 2014; Hanshew et al., 2015) and is well established in fungus-growing ants (Currie et al., 1999; Haeder et al., 2009; Oh et al., 2009; Barke et al., 2010; Poulsen et al., 2010; Schoenian et al., 2011; Carr et al., 2012; see Kaltenpoth and Engl, 2014 for a review).

Despite the general growth inhibition of ant body parts against *Metarhizium* sp., the supernatant of the ant's thorax and gaster tended to support fungal growth of *Metarhizium* sp. in comparison to the supernatant of the head (Fig. 3A). A similar pattern across ant body parts was seen for the growth of fungal associates, i.e. the capnodialean fungus (Fig. 3B), and the fungi in a solution derived from the carton-nest (Fig. 3C). Interestingly, the growth of the fungal associates was not only not inhibited but strongly favoured upon incubation with the supernatant of the thorax and the gaster compared to the supernatant of the head, and to the growth without the supernatant of ant body parts. Several non-exclusive explanations might account for this growth pattern across ant body parts. The presence of general nutrients in the thorax and gaster of *L. fuliginosus* ants might support the growth of fungal associates and antagonistic fungi, despite the general growth inhibition observed for *Metarhizium* sp. when incubated with *L. fuliginosus* ant body parts. In addition, *L. fuliginosus* ants have been observed to nourish fungi in their carton-nest with sugary solutions from their social stomach, the crop (Maschwitz and Hölldobler, 1970). As the crop is located in the ant gaster, specialized nutrients in the form of stored sugary solutions in the crop might be responsible for the increased growth of fungal associates. Alternatively, the growth of the capnodialean fungus and other fungi in the fungal solution of the carton-nest might have been favoured due to their ability to metabolize chemical compounds found in ant exocrine glands, in contrast to an overall growth inhibition of the antagonistic fungus *Metarhizium* sp. through these compounds. Indeed, it has been proposed that, especially, chaetothyrialean fungi might be predisposed to occupy ant nests because of their capacity to metabolize various chemical substances (Prenafeta-Boldú et al., 2002; Zhao et al., 2010; Voglmayr et al., 2011). Taken together, although we did not test specific chemical substances, the results of our growth assay are readily explained by the action of ant chemicals that inhibit antagonistic fungi, i.e. *Metarhizium* sp., but are tolerated by fungal associates and potentially even stimulate their growth. Further explicit testing of the source and contribution of ant chemicals and nutrients in inhibiting or stimulating the growth of fungal antagonists and associates will be needed to corroborate and extend our findings. Fungal growth assays with replicates of the same fungal isolates and of ants from the same nest were intended as a proof of principle experiment for the potential management of fungal associates via ant derived substances and to serve as a template for more detailed investigation. The use of several different fungal strains and ants from different *L. fuliginosus* colonies will be required for robust estimation of the extend and variability of growth effects via ant chemicals and nutrients.

By embracing a metacommunity approach and acknowledging the possibility of active management of microbial communities by the ants, we found a stable relationship between *L. fuliginosus* and fungal associates in carton-structures of their nest, which is likely shaped by host secretions. Future studies will have to determine whether ants that cultivate fungi in domatia and carton-nests in general have the potential to chemically control fungal and bacterial partners and, although very likely, whether this potential is also realised in the nest.

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Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.funeco.2018.08.007.

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