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# Social Organization and Subcaste Specialization in the Leaf-Cutting Ant *Acromyrmex subterraneus* (Formicidae: Myrmicinae)

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**Abstract** Several worker subcastes may occur in ant colonies, including physiological, morphological, and temporal subcastes. Leaf-cutting ants present intricate division of labor among worker subcastes during brood care, fungus garden maintenance, substrate foraging and processing. For colony survival, the fungus garden must be healthy, and tasks efficiently shared among worker subcastes. Therefore, worker behavior is key for colony maintenance in fungus-farming ants. Here we provide a qualitative and quantitative account of intracolony behavior in *Acromyrmex subterraneus*, a common leaf-cutter in Brazilian Cerrado savanna. Quantitative ethograms showed that performance of major behavioral categories (e.g., “Brood and Queen Care,” “Foraging,” “Fungus Care”) and the composition of behavioral repertoires are important parameters distinguishing labor among *A. subterraneus* worker subcastes. Media and major subcastes are behaviorally more similar to one another than to minors. Minors regularly executed brood- and fungus-related tasks,

whereas media and majors executed mostly foraging-related tasks. Grooming was frequent in all subcastes. Overall, the behavioral patterns reported in *A. subterraneus* are similar to those reported for other leaf-cutters. The tasks executed by different subcastes of *A. subterraneus* closely resemble the division of labor observed in *Atta* colonies, suggesting that alongside the use of fresh leaves as culturing substrates, a highly conserved set of worker behaviors persist since the origin of the leaf-cutting lineage. Our work highlights the importance of detailed analyses of the composition of behavioral repertoires in polymorphic fungus-farming ants to better understand their social organization, and the mechanisms mediating division of labor among worker subcastes in the Attina.

**Keywords** Cerrado savanna · division of labor · ethogram · fungus-farming ants · intracolony behavior · social behavior

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## Introduction

The eusocial mode of life with intracolony division of labor associated with phenotypic plasticity is considered one of the main attributes accounting for the ants' ecological success (Dupuis and Harrison 2017; Gordon and Traniello 2018). Such discrete phenotypes can be physiological, genetic, temporal, and morphological, and are frequently used to group individuals in different castes (Wilson 1971; Anderson and McShea 2001; Julian and Fewell 2004). In social insects, the term caste can

broadly designate the persistent physiological differences between reproductives and non-reproductives (i.e., physiological castes), as well as the allocation of workers into several tasks based on their age and/or morphology (Wilson 1971). Polymorphism within the worker caste characterize physical subcastes, which mediate task specialization and are part of the colony's plasticity (Wheeler 1986, 1991). For instance, in colonies of *Pheidole* ants (Myrmicinae), minor workers remain in the nest or forage in its proximities, whereas major workers primarily specialize for defense and/or subjugation and transport of large prey (Seid and Traniello 2006, and included references). Due to generation overlap, task allocation within the ant colony may also change accordingly with an individual's age, a phenomenon known as age polyethism (Wilson 1971). For instance, while young *Camponotus* workers (Formicinae) act mostly as nurses inside the nest, older workers typically engage in foraging and refuse dump management (Tripet and Nonacs 2004, and included references). Given that tasks outside the nest increase mortality risk by exposing workers to predators and pathogens, allocation of older workers to external labor seems to be adaptively advantageous to colonies (Hölldobler and Wilson 1990). Thus, division of labor and task allocation within the colony can be mediated by worker size, age and experience, as well as by social interactions among colony members and their environment (see Gordon 2016; Jeanne 2016).

Leaf-cutting ants illustrate how temporal and physical worker subcastes function together to execute tasks that maintain both the structure and sanitary conditions of the nest and symbiont fungus (Wilson 1980a; Camargo et al. 2007). Also known as fungus-farming ants (Myrmicinae, Attini, Attina; hereafter “attine” ants), they cultivate a symbiont fungus inside the nest and use it as their main food source. Attine ants may collect a variety of items for fungiculture, including arthropod feces, plant detritus, insect corpses, and fresh plant parts (Leal and Oliveira 1998; Mehdiabadi and Schultz 2009; Ronque et al. 2019). During the evolutionary history of fungus-farming ants, the highly specialized genera *Acromyrmex* and *Atta* acquired the habit of actively cutting fresh plant matter as substrate for fungus culturing. In the nest, the culturing substrate undergoes a meticulous processing by workers before assimilation by the symbiont fungus – the “leaf-cutter agriculture” (Schultz and Brady 2008). While major workers attack predators and assist during the cutting

of thick leaves, medium-sized workers act as main foragers by cutting and transporting culturing material back to the nest. In the nest, minor workers clip plant material into smaller fragments, crush and mold them into moist pellets, and insert them in the fungus garden (Hölldobler and Wilson 2011). To ensure the welfare of the fungus, the culturing substrate undergoes continuous cutting and cleaning by ants of different sizes, whereas caretakers regularly patrol the fungus garden to keep it free from harmful microorganisms – if the symbiont fungus perishes, the colony dies (Currie et al. 1999). Therefore, the capacity to keep the nest environment free of pathogens and properly nurture the symbiont is crucial for colony survival, requiring effective and constant labor by each worker subcaste (Currie et al. 1999; Currie and Stuart 2001).

*Acromyrmex* species present two to four worker subcastes, and division of labor is mediated by an interplay of morphological and temporal subcastes (Wetterer 1999; Camargo et al. 2007). Here, we provide a qualitative and quantitative account of the intracolony behavioral pattern of *Acromyrmex subterraneus* (Fig. 1), a common leaf-cutter ant in native Cerrado savanna of Brazil. We used captive colonies to investigate how intracolony behaviors differ among worker subcastes, and how the observed pattern compares to the behavioral organization observed in other attines (Wilson 1980a; Beshers and Traniello 1996, Ronque 2018). We describe the behavioral repertoire and produce quantitative ethograms of the worker subcastes for three *A. subterraneus* colonies. We hypothesized that the intracolony behavioral pattern of *A. subterraneus* will be in accordance with the necessities imposed by the ant-fungus mutualism, that is, minor workers will most likely perform fungus-related behaviors, whereas media and major workers will most often execute foraging-related tasks.

## Materials and Methods

### Study Site

Colonies were collected in a 470-ha Cerrado reserve near Mogi-Guaçu, state of São Paulo, Southeast Brazil (22°18'S, 47°11'W). The vegetation consisted of a forest-like ‘cerradão’, formed by 50 to 90% of trees up to 10–12 m in height (Oliveira-Filho and Ratter 2002). The characteristic climate of the region presents a dry/



**Fig. 1** Close-up view of the fungus garden of a captive colony of *Acromyrmex subterraneus*, illustrating the diversity of substrates used for fungus culturing, and a media worker depositing recently-collected substrate on the garden. Photograph courtesy of Hélio Soares Jr.



cold season (winter) from April to September and a rainy/hot season (summer) from October to March.

#### Collection and Maintenance of Ant Colonies

Colonies of *A. subterraneus* ( $N = 3$  nests) were collected during the dry season, and were located in the field by active searching the characteristic nest mounds and foraging trails of this species. Tagged nests had only one entrance and were at least 20 m apart from one another. After locating a nest, a rectangular trench ( $1.0 \times 1.5$  m) was dug and excavations began 0.5 m distant from the nest entrance. Excavations were 2 m deep below the ground surface, until the chamber containing fungus garden was located. All observed workers, the fungus garden, the queen, and the brood were collected and placed in a clean plastic container (see Sosa-Calvo et al. 2015). Once collected, all organisms were transported on the same day to the laboratory at the Universidade Estadual de Campinas. Ant voucher specimens are deposited at the “Museu de Zoologia da Universidade Estadual de Campinas,” São Paulo (ZUEC, Campinas, Brazil; registration number 6261).

Colonies were housed in artificial nests consisting of three glass boxes connected by transparent plastic tubes. The first box ( $10 \times 10$  cm; 6 cm high) contained culturing material (fresh leaves and flowers) and was designated as the “foraging arena;” the second box ( $13 \times 13$  cm; 8.5 cm high) was lined with 1-cm-thick moist plaster layer to maintain humidity, and was assigned as the “fungus garden chamber.” The third box ( $10 \times 10$  cm; 6 cm high) contained discarded material such

as exhausted fungi, dead nestmates, and unused culturing substrate, and was labelled as the “refuse dump.” Captive colonies were cultured at 25 °C and 80% relative humidity, under a 12:12 h lighting regimen (artificial light from 07:00 to 20:00 h). Colonies were provided *ad libitum* with culturing substrate and water (moistening of plaster floor) three times a week. Substrate for fungus culturing included fresh leaves of *Acalypha* (Euphorbiaceae) and fresh flowers of *Bauhinia* (Fabaceae), *Rosa* (Rosaceae), and *Hibiscus* (Malvaceae).

#### Behavioral Observations

Three colonies of *A. subterraneus* were used to produce the ethograms. This sample size was considered adequate since many similar studies with ants (including attines) utilized three or less colonies (e.g., Wilson 1976; Herbers 1983; Moffett 1986; Villet and Wildman 1991; Pratt et al. 1994; Pie 2002; Della Lucia et al. 2003; Santos et al. 2005; Camargo et al. 2007, Vieira et al. 2010), and more rarely four or five colonies (e.g., Brandão 1983; Beshers and Traniello 1996). Ants were allowed to habituate to the laboratory conditions for one week. Morphological worker subcastes were visually categorized by head width into minor (0.7–1.1 mm), media (1.2–1.6 mm) and major workers (1.7–2.0 mm), following the size distribution used by Camargo et al. (2007). Although the ants were not marked, worker size categories were easily distinguishable visually (Figure S1 in Online Resource 1). Three queenright colonies ( $\approx 1100$  to 1700 workers) were used to gather

behavioral data of *A. subterraneus*. Although mature colonies of *A. subterraneus* colonies have been reported to contain 10 to 20 thousand individuals (Pereira and Della Lucia 1998), we consider the size of the colonies used in this study big enough to sample appropriately the workers' behavioral repertoires. Previous behavioral studies with *A. subterraneus* utilized captive colonies containing ca. 800 to 1000 workers (Forti et al. 2004).

Seven hours of qualitative observations per colony, during a 7-week observation period, provided the list of behavioral acts and categories applied for the quantitative samplings (Table 1; see also Table S1 in Online Resource 1, for descriptions of behavioral acts). Functionally related behavioral acts were grouped in the same major behavioral category. For instance, all behaviors related to the care and welfare of queen and brood, such as antennating or licking them, were grouped under "Brood and Queen Care" (Table 1). Ethogram data were taken in intermittent 1 h sessions during the normal activity period of the ants, totaling 7 h of observation per subcaste per colony (21 h per colony), during a 17-week observation period. Quantitative ethograms were produced by scan samplings (Altmann 1974; Lehner 1996). Each scan lasted 10 min; after a 5 min interval a new scanning was made, and so on. Calculation of sampling coverage per colony and subcaste followed Fagen and Goldman (1977), according to the formula:

$$\Theta = 1 - \left( \frac{N_1}{i} \right)$$

Where "Θ" is the sampling coverage, "N<sub>1</sub>" is the number of behavioral acts observed only once, and "i" the total of acts observed. Values of Θ between 0.90 and 1 indicate that sampling has reached sufficiency. The closer Θ approaches 1, the probability of observing a new behavioral act is low, and the more complete the sample coverage (Fagen and Goldman 1977).

The relative frequency of each behavioral act per subcaste per colony was calculated by dividing the number of times the act was performed by the total acts performed by the subcaste (Wilson 1976). Behavioral acts that were functionally related were grouped into nine major behavioral categories as follows (Table 1): "Brood and Queen Care," "Foraging," "Fungus Care," "Grooming," "Interaction between workers," "Interaction with Refuse Dump and Dead Workers," "Locomotion," "Other Activities," and "Stationary"

(see also Wilson 1976; Murakami and Higashi 1997; Ronque 2018). "Grooming" was quantified due to its importance in colonies of fungus-growing ants, since this behavior prevents the presence and spread of pathogens that can affect the ants and the fungus garden (Currie et al. 1999; Hölldobler and Wilson 2011). Therefore, it is expected that the subcaste most frequently in contact with the fungus would also self-groom and be groomed more often to prevent contamination (Abramowski et al. 2011). The category "Locomotion" was established because tasks related with the processing of culturing substrates and maintenance of the colony are executed sequentially throughout the nest (Mehdiabadi and Schultz 2009). Accordingly, the presence of a given worker subcaste in a nest compartment (i.e., the subcaste spatial distribution) could be associated with the execution of tasks frequently associated with that specific compartment (i.e., a subcaste that frequently walks in the fungus-garden chamber would also more frequently execute tasks related to "Fungus Care"). "Stationary" behavior was considered for the repertoire of *A. subterraneus* workers because idleness has previously been reported as a common trait in major and super major worker subcastes of *Atta* species (Hölldobler and Wilson 2011). Additionally, there is evidence that long-term colony sustainability may require that some individuals remain idle to recover from energetically costly activities (Hasegawa et al. 2016). Therefore, it is expected that the workers in the subcaste performing more costly labor would also be stationary more often.

## Statistical Analyses

All analyses were carried out using the R Software, version 3.5.0 (R Core Team 2018). We performed a Generalized Linear Mixed Model (*glmer* function, *lme4* package; Bates et al. 2015) with Poisson distribution to investigate variations in the frequency of behavioral categories among subcastes. Subcastes and behavioral categories acted as the main explanatory variable (fixed factor) and colony of origin as a random factor. We performed pairwise comparisons with Tukey test (*lsmeans* package, function *lsmeans*; Lenth 2016) among the subcastes. Additionally, behavioral acts within the major categories "Foraging" and "Fungus Care" were analyzed using a Generalized Linear Mixed Model (*glmer* function, *lme4* package; Bates et al. 2015) with Poisson distribution to investigate variation in their

**Table 1** Behavioral repertoire and quantitative ethogram of the three studied colonies of the fungus-farming ant *Acromyrmex subterraneus*. Functionally related behavioral acts are grouped within major behavioral categories. The table provides the absolute value of each behavior, and the relative frequency (%) in parentheses. A hifen (–) designates the behavior was not observed, and 0 indicates that the behavior was observed during the qualitative observations but not during the quantitative sampling. N indicates the initial number of individuals in each colony. Sampling coverage (Θ) follows Fagen and Goldman (1977); the closer Θ approaches 1, the more complete the sample coverage

Behavioral Categories	Colony 1 (N = 1105)				Colony 2 (N = 1200)				Colony 3 (N = 1717)				
	Minor	Media	Major	Total	Minor	Media	Major	Total	Minor	Media	Major	Total	
<i>Brood and Queen Care</i>													
Antennate Queen	0	0	0	0	0	3(0.2)	0	0	3(0.1)	2(0.1)	0	0	2(0.04)
Lick Queen	0	0	0	0	0	2(0.1)	–	–	2(0.04)	–	–	–	–
Manipulating Brood	17(1.1)	9(0.5)	6(0.4)	32(0.7)	18(1.1)	18(1.1)	2(0.1)	4(0.2)	24(0.5)	6(0.4)	2(0.1)	1(0.1)	9(0.2)
(Total score in the category)	17(1.1)	9(0.5)	6(0.4)	32(0.7)	23(1.4)	23(1.4)	2(0.1)	4(0.2)	29(0.6)	8(0.5)	2(0.1)	1(0.1)	11(0.2)
<i>Foraging</i>													
Manipulate and Cut Substrate	70(4.3)	177(10.3)	106(7.3)	353(7.4)	212(12.8)	212(12.8)	320(21.5)	318(18.4)	850(17.5)	29(1.9)	279(17.4)	265(17.8)	573(12.4)
Transport Substrate	12(0.8)	57(3.3)	25(1.7)	94(2.0)	26(1.6)	26(1.6)	101(6.8)	99(5.7)	226(4.6)	9(0.6)	81(5.1)	67(4.5)	157(3.4)
(Total score in the category)	82(5.1)	234(13.6)	131(9.0)	447(9.4)	238(14.4)	238(14.4)	421(28.3)	417(24.2)	1076(22.1)	38(2.5)	360(22.5)	332(22.4)	730(15.8)
<i>Fungus Care</i>													
Manipulate Fungus	154(9.6)	57(3.3)	76(5.2)	287(6.0)	228(13.8)	228(13.8)	78(5.2)	86(5.0)	392(8.1)	416(27.0)	86(5.4)	97(6.5)	599(12.9)
Transport Fungus	33(2.0)	6(0.4)	1(0.1)	40(0.8)	30(1.8)	30(1.8)	12(0.8)	8(0.5)	50(1.0)	64(4.1)	25(1.6)	8(0.5)	97(2.1)
(Total score in the category)	187(11.6)	63(3.7)	77(5.3)	327(6.8)	258(15.6)	258(15.6)	90(6.0)	94(5.5)	442(9.1)	480(31.1)	111(7.0)	105(7.1)	696(15.0)
<i>Grooming</i>													
Allogrooming	98(6.1)	72(4.2)	15(1.0)	185(3.9)	156(9.4)	156(9.4)	34(2.3)	13(0.8)	203(4.2)	31(2.0)	61(3.8)	14(0.9)	106(2.3)
Self-grooming	227(14.1)	284(16.5)	228(15.7)	739(15.5)	204(12.3)	204(12.3)	234(15.7)	341(19.8)	779(16.0)	258(16.7)	305(19.1)	283(19.1)	846(18.3)
(Total score in the category)	325(20.2)	356(20.7)	243(16.7)	924(19.3)	360(21.7)	360(21.7)	268(18.0)	354(20.5)	982(20.2)	289(18.7)	366(22.9)	297(20.0)	952(20.6)
<i>Interaction Between workers</i>													
Antennate Nestmate	97(6.0)	158(9.2)	108(7.4)	363(7.6)	50(3.0)	50(3.0)	87(5.8)	65(3.8)	202(4.1)	95(6.2)	99(6.2)	75(5.1)	269(5.8)
Bite Nestmate	2(0.1)	3(0.2)	1(0.1)	6(0.1)	2(0.1)	2(0.1)	1(0.1)	0	3(0.1)	0	2(0.1)	0	2(0.04)
Lick Dying Nestmate	–	–	–	–	–	–	–	–	–	–	1(0.1)	0	2(0.04)
Transport Nestmate	0	3(0.2)	0	3(0.1)	1(0.1)	1(0.1)	2(0.1)	2(0.1)	5(0.1)	1(0.1)	0	0	1(0.02)
Trophallaxis	18(1.1)	44(2.6)	4(0.3)	66(1.4)	17(1.0)	17(1.0)	24(1.6)	14(0.8)	55(1.1)	15(1.0)	31(1.9)	18(1.2)	64(1.4)
(Total score in the category)	117(7.3)	208(12.1)	113(7.8)	438(9.2)	70(4.2)	70(4.2)	114(7.7)	81(4.7)	265(5.4)	112(7.3)	133(8.3)	93(6.3)	338(7.3)
<i>Interaction with Refuse Dump and Dead Workers</i>													
Manipulate Refuse Dump	66(4.1)	19(1.1)	4(0.3)	89(1.9)	22(1.3)	22(1.3)	19(1.3)	6(0.4)	47(1.0)	76(4.9)	44(2.8)	4(0.3)	124(2.7)
Manipulate Dead Worker	24(1.5)	23(1.3)	6(0.4)	53(1.1)	31(1.9)	31(1.9)	3(0.2)	2(0.1)	36(0.7)	0	1(0.1)	0	1(0.02)
(Total score in the category)	90(5.6)	42(2.4)	10(0.7)	142(3.0)	53(3.2)	53(3.2)	22(1.5)	8(0.5)	83(1.7)	76(4.9)	45(2.8)	4(0.3)	125(2.7)

**Table 1** (continued)

Behavioral Categories	Colony 1 (N = 1105)			Colony 2 (N = 1200)			Colony 3 (N = 1717)					
	Minor	Media	Major	Total	Minor	Media	Major	Total	Minor	Media	Major	Total
<i>Locomotion</i>												
Walking in Foraging Arena	121(7.5)	139(8.1)	155(10.7)	415(8.7)	97(5.9)	116(7.8)	162(9.4)	375(7.7)	2(0.1)	71(4.4)	38(2.5)	111(2.4)
Walking in Fungus Chamber	147(9.1)	139(8.1)	172(11.8)	458(9.6)	113(6.8)	70(4.7)	151(8.7)	334(6.9)	150(9.7)	105(6.6)	112(7.5)	367(7.9)
Walking in Plastic Tube	0	0	0	0	0	1(0.1)	3(0.2)	4(0.1)	2(0.1)	7(0.4)	4(0.3)	13(0.3)
Walking in Refuse Dump	119(7.4)	106(6.2)	56(3.9)	281(5.9)	64(3.9)	55(3.7)	43(2.5)	162(3.3)	48(3.1)	50(3.1)	1(0.1)	99(2.1)
Walking over Fungus	80(5.0)	54(3.1)	94(6.5)	228(4.8)	99(6.0)	34(2.3)	60(3.5)	193(4.0)	142(9.2)	61(3.8)	103(6.9)	306(6.6)
Walking over Substrate	4(0.3)	15(0.9)	20(1.4)	39(0.8)	38(2.3)	66(4.4)	53(3.1)	157(3.2)	4(0.3)	86(5.4)	44(3.0)	134(2.9)
(Total score in the category)	471(29.3)	453(26.4)	497(34.3)	1421(29.7)	411(25.0)	342(23.0)	472(27.4)	1225(25.2)	348(22.6)	380(23.7)	302(20.3)	1030(22.2)
<i>Other Activities</i>												
Antennate Nest Parts	12(0.7)	18(1.0)	16(1.1)	46(1.0)	1(0.1)	3(0.2)	8(0.5)	12(0.2)	13(0.8)	12(0.7)	3(0.2)	28(0.6)
Biting and Licking Nest Parts	90(5.6)	36(2.1)	16(1.1)	142(3.0)	47(2.8)	17(1.1)	13(0.8)	77(1.6)	85(5.5)	56(3.5)	17(1.1)	158(3.4)
Collect Fecal Secretion	1(0.1)	0	0	1(0.02)	0	0	0	0	0	0	0	0
Lick Fecal Pellet	0	0	1(0.1)	1(0.02)	1(0.1)	0	0	1(0.02)	0	0	0	0
Mandibles Wide Open	–	–	–	–	0	1(0.1)	1(0.1)	2(0.04)	0	1(0.1)	11(0.7)	12(0.3)
Stridulation	65(4.0)	36(2.1)	10(0.7)	111(2.3)	56(3.4)	34(2.3)	11(0.6)	101(2.1)	25(1.6)	22(1.4)	12(0.8)	59(1.3)
(Total score in the category)	168(10.4)	90(5.2)	43(3.0)	301(6.3)	105(6.3)	55(3.7)	33(1.9)	193(4.0)	123(8.0)	91(5.7)	43(2.9)	257(5.6)
<i>Stationary</i>												
Total Number of Performed Acts	153(9.5)	264(15.4)	331(22.8)	748(15.7)	139(8.4)	174(11.7)	263(15.2)	576(11.8)	68(4.4)	113(7.1)	308(20.7)	489(10.6)
Total of Described Acts	1610	1719	1451	4780	1657	1488	1726	4871	1542	1601	1485	4628
Total of Categories	23	23	23	25	26	25	24	28	24	25	22	27
Sampling Coverage (€)	0.9994	1	0.9979	0.9996	0.9982	0.9980	0.9994	0.9998	0.9987	0.9981	0.9987	0.9996



frequencies among subcastes in each of these categories. Subcastes and behavioral acts acted as the main explanatory variable (fixed factor) and colony of origin as a random factor. We also performed pairwise comparisons with Tukey test (*lsmeans* package, function *lsmeans*; Lenth 2016) among the subcastes in relation to the frequency of each behavioral acts.

PERMANOVA analysis, performed with the *vegan* package (*adonis* function; Oksanen et al. 2019), quantified the compositional similarities between behavioral repertoires among subcastes via Bray-Curtis dissimilarity. We performed principal coordinates analysis (PCoA), using Bray-Curtis dissimilarity (*ape* package, function *pcoa*; Paradis and Schliep 2018), to graphically visualize differences in the behavioral repertoire among subcastes. Additionally, we identified the major behavioral categories responsible for the differences in behavioral repertoires among subcastes with Similarity percentage analysis (SIMPER), performed using Bray-Curtis dissimilarity (*vegan* package; *simper* function; Oksanen et al. 2019). SIMPER showed the contribution of each behavioral category to the dissimilarity among minor, media and major subcastes. Lastly, we generated the Heatmap of the behavioral acts with the package “*gplots*” (function *heatmap.2*; Warnes et al. 2005). The Heatmap’s dendrogram was constructed using Bray-Curtis dissimilarity (*vegan* package, function *vegdist*; Oksanen et al. 2019). For clearer visualization of the relative frequencies of each behavioral act, we removed acts with less than 1% of relative frequency.

## Results

### General Behavioral Patterns by Worker Subcastes

The behavioral catalog and quantitative ethogram of *A. subterraneus* worker subcastes are presented in Table 1 for three queenright colonies. Overall, major behavioral categories were performed in different frequencies ( $\text{Chisq} = 1836.17$ ;  $p < 0.001$ ;  $\text{df} = 8$ ); the most executed categories were “Foraging,” “Fungus Care,” “Grooming,” “Locomotion,” and “Stationary” (Fig. 2a; Table 1). Ethogram data per subcaste revealed that major behavioral categories were performed in different frequencies among minor, media, and major workers ( $\text{Chisq} = 1400.07$ ;  $p < 0.001$ ;  $\text{df} = 16$ ; Fig. 2b). Pairwise comparisons revealed differences between worker subcastes in the following major behavioral

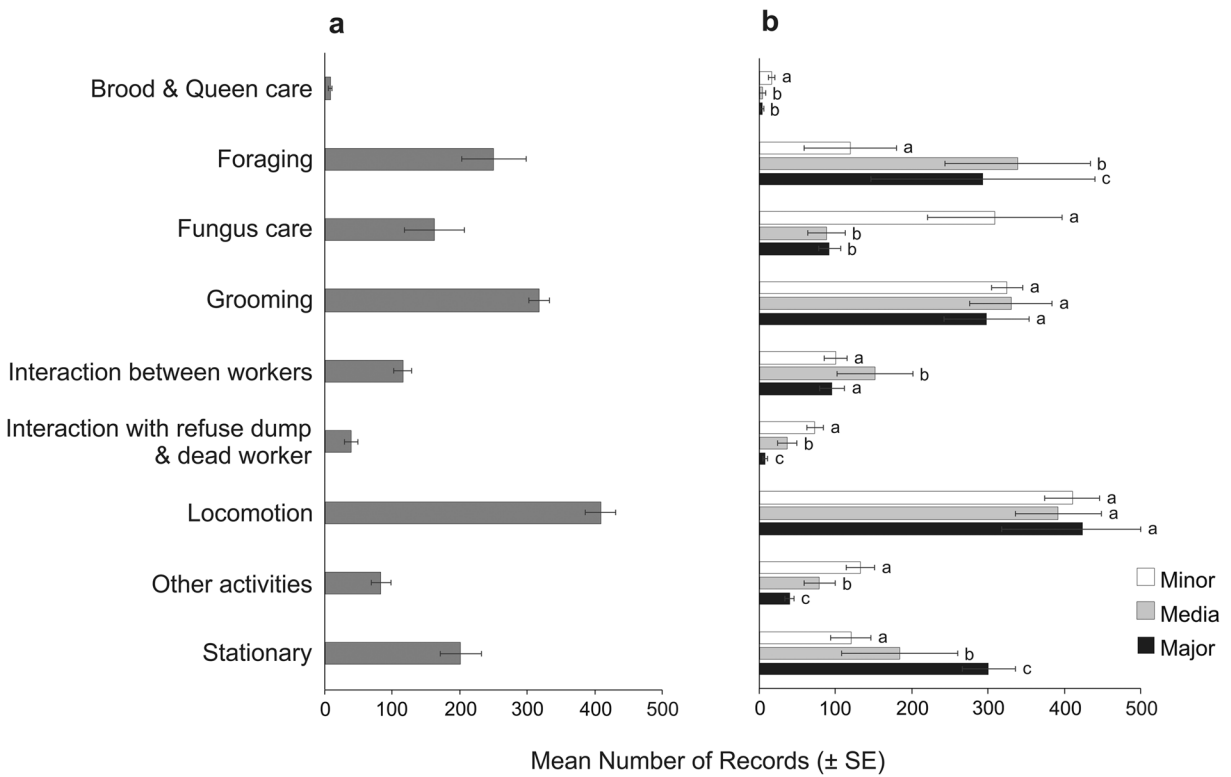
categories: “Brood and Queen Care,” “Foraging,” “Fungus Care,” “Interactions between workers,” “Interaction with Refuse Dump and Dead Workers,” “Other Activities,” and “Stationary” (Fig. 2b; Table S2 in Online Resource 1).

Some behavioral categories such as “Foraging” and “Fungus Care” are intrinsically related to division of labor. For instance, the behavioral acts “Manipulate and Cut Substrate” and “Transport Substrate” are related tasks in the category “Foraging.” Similarly, “Manipulate fungus” and “Transport Fungus” are associated in the category “Fungus Care.” Notwithstanding, behavioral acts altogether were performed in different frequencies among worker subcastes, both in the categories “Foraging” ( $\text{Chisq} = 18.68$ ;  $p < 0.01$ ;  $\text{df} = 2$ ; Fig. S2 in Online Resource 1) “Fungus Care” ( $\text{Chisq} = 13.68$ ;  $p < 0.01$ ;  $\text{df} = 2$ ; Fig. S3 in Online Resource 1).

The composition of the behavioral repertoire differed among the subcastes (PERMANOVA,  $\text{df} = 2$ , pseudo- $F = 4.15$ ,  $R^2 = 0.58$ ,  $p = 0.03$ ). The PCoA (Fig. 3) shows that each subcaste had a distinct behavioral repertoire and the differences among them are characterized with the first two axes, the first axis explained 61% of the differences and the second explained 23%. The SIMPER analysis (Table 2) revealed that the behavioral categories contributing to the dissimilarity between (1) minor/media subcastes were “Foraging,” “Fungus Care,” “Locomotion,” and “Stationary,” (2) minor/major were “Fungus Care,” “Foraging,” “Stationary,” and “Other Activities;” (3) media/major worker subcastes were “Foraging,” “Grooming,” “Locomotion,” and “Stationary.” Additionally, the SIMPER analysis revealed that the average dissimilarity in the behavioral repertoire between minor and media workers was 24%, between minor and major workers was 30%, and between media and major workers was 17% (Table 2). That is, media and major subcastes had the most similar behavioral repertoires.

### Behavioral Acts and Worker Subcastes

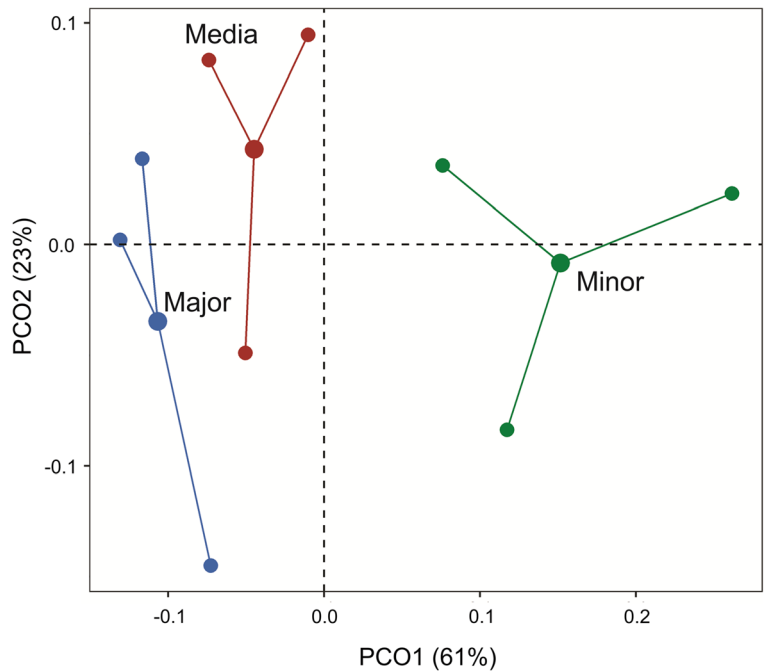
“Manipulate Fungus” was by far the most frequent ( $\approx 16\%$ ) behavioral act performed by workers in the minor subcaste (Table 1, Fig. 4). On the other hand, the most frequent behaviors by media and major workers ( $\approx 16$  to  $23\%$ ) were “Self-grooming,” “Manipulate Substrate,” and “Stationary” (Table 1, Fig. 4). The Heatmap and dendrogram clustered media and major workers, separating these subcastes from minor workers (Fig.4).



**Fig. 2** Occurrence of major behavioral categories based on quantitative ethograms of three captive colonies of *Acromyrmex subterraneus*, taking into account (A) all sub-castes and (B) each

sub-caste (different letters denote significant statistical difference). Bars indicate mean ± SE. See also Table 1, and Table S2 from Online Resource 1

**Fig. 3** Two-dimensional plot of principal coordinates analysis (Bray-Curtis distance) showing differences in the composition of the behavioral repertoire by the three subcastes of *Acromyrmex subterraneus*. Each colony observed (N = 3) is represented by the minor circles; the centroids are designated by bigger circles. See also Table 2



**Table 2** Similarity percentage analysis (SIMPER) among subcastes of *Acromyrmex subterraneus* per behavioral category. The lower a percent value for a given behavioral category, the higher its contribution to dissimilarity between subcastes

Comparison between subcastes	Behavioral Category	Cumulative Contribution (%)
Minor vs Media Average dissimilarity (%): 24	Fungus Care	57.09
	Foraging	28.66
	Stationary	67.05
	Locomotion	74.53
Minor vs Major Average dissimilarity (%): 30	Fungus Care	23.61
	Foraging	44.80
	Stationary	64.53
	Other Activities	74.52
Media vs Major Average dissimilarity (%): 17	Foraging	22.68
	Stationary	44.96
	Locomotion	62.23
	Grooming	73.07

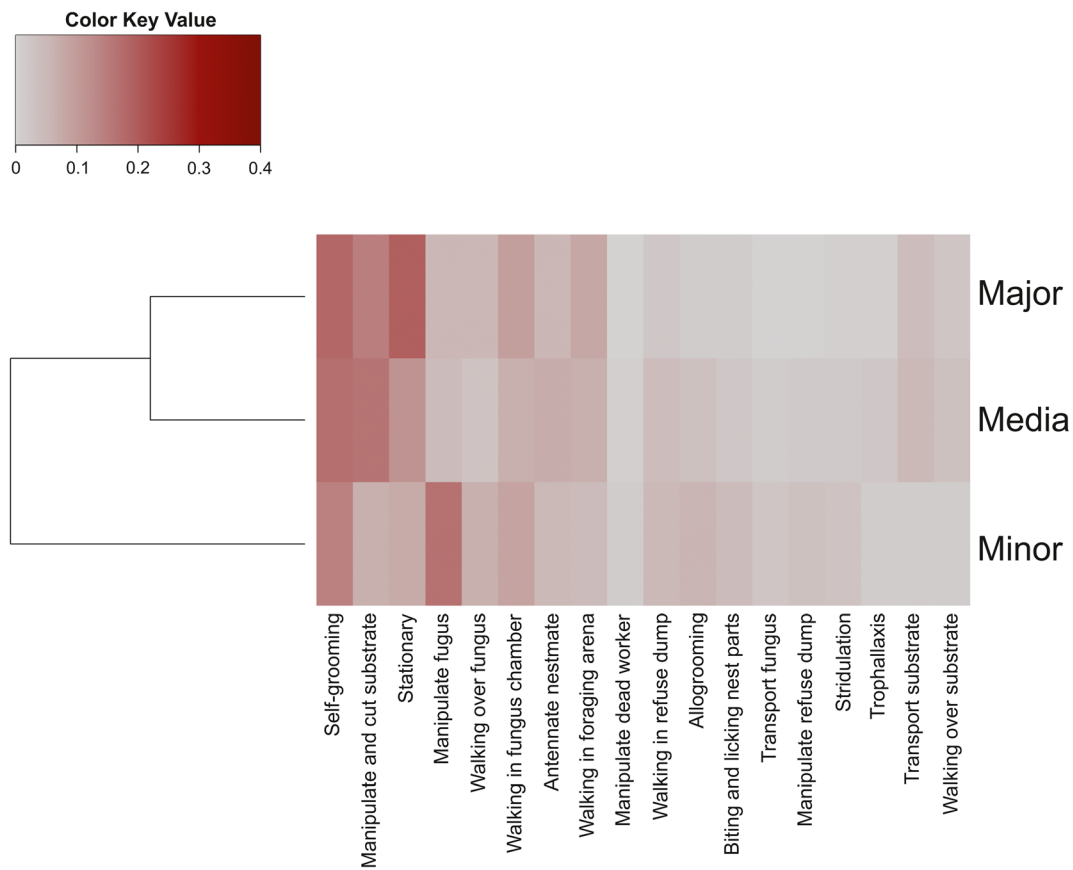
This indicates that media and major workers were more similar to one another regarding the frequency of particular behavioral acts, most notably the manipulation of culturing substrate and inactivity.

## Discussion

Our ethogram data show that intracolony behavior differs significantly among worker subcastes of *A. subterraneus*. Minor, media and major workers differed in the allocation of labor to three general behavioral categories, “Foraging,” “Fungus Care,” and “Stationary.” Overall, media and major subcastes are behaviorally more similar to one another than to minors. Our study shows that behavioral differentiation among worker subcastes occurs not only in the frequency of particular behavioral acts, but also in composition of the behavioral repertoire, as a whole, by each subcaste. Such behavioral distinction among worker subcastes could result from variable developmental pathways leading to labor specialization by each group (Wheeler 1986, 1991; Corona et al. 2016).

*Acromyrmex* species present two to four subcastes (Wetterer 1999; Forti et al. 2004; Camargo et al. 2007), and our behavioral analysis in this study confirmed the existence of three well-distinguished subcastes within colonies of *A. subterraneus*. The observed behavioral repertoire consisted of 9 major behavioral categories containing a total of 29 behavioral acts (Table 1), which

is consistent with the behavioral catalogues reported by Camargo et al. (2007) and Wilson (1980a) for *A. subterraneus brunneus* and *Atta sexdens*, respectively, each comprising 29 behavioral acts. The breadth of our behavioral repertoire, however, is below the range (37 to 49 behavioral acts) recorded by Ronque (2018) for five species of non-leaf-cutter attines of the Brazilian Atlantic rainforest (genera *Mycocepurus*, *Mycetarotes*, *Mycetophylax*, and *Sericomyrmex*), and by Beshers and Traniello for *Trachymyrmex septentrionalis* (36 behavioral acts). Discrepancy in the number of behavioral acts observed in different studies may result from distinct social structures among fungus-farming ants. In leaf-cutting ants, different groups of workers tend to allocate efforts to different tasks, which are executed simultaneously (Wilson 1980a; Forti et al. 2004; Camargo et al. 2007; Mehdiabadi and Schultz 2009). While a group of ants cut and/or transport culturing substrates in the outside environment, in the nest other ants process the foraged material and cultivate the fungus garden or manipulate the contents of the refuse dump. Such simultaneous task execution in broad scale as seen in colonies of leaf-cutting ants is attainable due to their huge numbers, ranging from thousands to millions of workers (Hölldobler and Wilson 2011). Since colonies of non-leaf-cutting, fungus-farming ants are far less numerous than typical leaf-cutter species, fewer workers have to execute all tasks associated with fungus culturing, which likely broadens the workers’ behavioral spectrum as recorded by Beshers and Traniello (1996) and



**Fig. 4** Heatmap indicating variation in the relative frequency of behavioral acts performed by the subcastes of three colonies of the fungus-farming ant *Acromyrmex subterraneus*. Color variation indicates the relative frequency of the behavioral acts performed,

ranging from 0% (grey areas) to 40% (dark areas). Dendrograms were generated using the Bray-Curtis distance matrix method. Behavioral acts with relative frequency lower than 1% were not considered

Ronque (2018). These findings reinforce the importance of natural history data of fungus-farming ants in relation to the activities executed both inside and outside the nest.

Locomotion, grooming, foraging, and fungus care were very frequent behaviors in the colonies of *A. subterraneus*. Indeed, there is intense traffic between nest compartments to perform the sequential tasks associated with fungus culture. For instance, foragers locate and cut culturing substrate in the foraging arena and transport these to the fungus garden chamber, where the material will be processed or discarded in the refuse dump (Garrett et al. 2016). This chain of events force individuals to move repeatedly between nest compartments, which make colonies more prone to disease spread due to constant contact between nestmates coming from or

going to different places (Waddington and Hughes 2010; Farji-Brener et al. 2016). As such, pathogens from inside and outside the nest environment can easily spread and make both workers and the symbiont fungus vulnerable to infections that could lead to death of the fungus, and of the entire colony due to starvation (Currie et al. 1999). Therefore, not only the culturing substrates carried into the nest need to be cleaned several times, but also workers need to constantly self-groom and be groomed by nestmates (Richard and Errard 2009). Allogrooming (social grooming), however, could act as a double-edged sword for colony immunity since allogroomers may also transfer pathogens to nestmates (Fefferman et al. 2007; Theis et al. 2015). In the case of *A. subterraneus*, grooming has been reported as an important mechanism of communication and

recognition in the colony by transferring surface substances (cuticle hydrocarbons) among nestmates, which homogenizes colony odor (Camargo et al. 2006). As such, both locomotion (associated with substrate processing) and grooming (associated with asepsis) are important behaviors for the maintenance of both the fungus symbiont and the ant colony.

Preventing the entry of pathogens in the nest and nurturing the fungus properly are not, however, enough to keep the symbiont healthy. It is necessary that worker caretakers closely monitor the fungus and constantly patrol it, cutting down and grooming areas infected with alien fungi or sickly symbiont hyphae (Currie and Stuart 2001). These behaviors hamper the spread of contaminants in the symbiont fungus, preventing its death. For instance, licking constitutes an important behavior in the “Fungus Care” category since leaf-cutters have antibiotics in their metapleural glands (Quinlan and Cherrett 1978; Poulsen et al. 2002). Therefore, by continuously licking and weeding the symbiont fungus, caretakers prevent bacterial contamination and dispatch them through infrabuccal pocket pellets (Quinlan and Cherrett 1978; Currie and Stuart 2001).

Our ethograms showed that minor workers are mostly concerned with tasks related with the grooming of brood and queen, and the maintenance of the fungus garden. The same behavioral pattern was observed previously in leaf-cutters, and tends to vary little with worker age (Wilson 1980a; Forti et al. 2004; Camargo et al. 2007). Due to their minute size, minor workers are able to manipulate and clean symbiont hyphae more easily than larger subcastes, reacting first to pathogenic microorganisms and invasive fungus, and to changes in the symbiont garden (Abramowski et al. 2011). The other subcastes are responsible for discarding the contaminated parts of the fungus in the refuse dump (Currie and Stuart 2001). Specialization of the minor subcaste for fungus care is further supported by the fact that their infrabuccal pockets are small and allow the filtering of minute particles from the symbiont surface (Quinlan and Cherrett 1978). The high frequency of brood and queen care by minors of *A. subterraneus* may also have to do with the fact that in this subcaste the behavioral repertoire changes little with age (Camargo et al. 2007), effectively making these individuals lifetime nurses. On the other hand, Beshers and Traniello (1996) suggested that minute size alongside young age, seem to be

key for adequate management of the fungus garden by workers of *Trachymyrmex septentrionalis*.

To our surprise, minor workers of *A. subterraneus* interacted with refuse dump and dead workers more frequently than media and major workers (Fig. 2b). Minor workers of leaf-cutter ants are known to hitchhike on larger nestmates, defending them from attack by parasitic phorid flies and cleaning the culturing material prior to entering the nest (Feener Jr. and Moss, 1990; Griffiths and Hughes 2010). Indeed, hitchhiking by minors was frequently observed in *A. subterraneus* foraging trails in the Cerrado reserve (Calheiros 2019). In captivity, however, because the distance between the foraging arena and the fungus garden was short, and there were no parasitic threats, hitchhiking behavior by minors was probably discouraged. Moreover, in captivity conditions substrate cleaning was performed directly at the foraging arena or at the fungus garden chamber. Thus, it appears that minor workers had their task allocation directed to a remaining perilous task in the artificial nest: management of the refuse dump and dead nestmates. This sanitary activity by minors could prevent the spread of pathogens within the nest environment.

Media and major workers of *A. subterraneus* stood out for the high frequency of both foraging and stationary behavior (Fig. 2b). Among leaf-cutter species, these subcastes are usually responsible for foraging, since the workers have large/strong mandibles that allow the manipulation/cutting of hard substrate for fungiculture (Wilson 1980a, b; Schofield et al. 2002, 2011). Media and major workers remain largely stationary probably because foraging is the most energetically costly task executed by leaf-cutting ants (Roces and Lighton 1995; Burd 1996). Indeed, leaf-cutters may take turns to switch the foraging effort and prevent excessive wear of the workers, as suggested by Hasegawa et al. (2016). In the case of *A. subterraneus*, Camargo et al. (2007) have shown that foraging tasks are performed mostly by the old workers, which could lead to further physiological strain of their bodies.

Most behavioral accounts on caste polymorphism and division of labor in leaf-cutting ants were carried out using *Atta* colonies as the study system (Hölldobler and Wilson 2011, and included references). Overall, the behavioral patterns reported here for *A. subterraneus* are similar to the results obtained for worker subcastes of



other ant species, including leaf-cutters: minors perform activities primarily inside the nest, medias deal mostly with foraging tasks, and majors help obtain/transport large items, and often remain idle (e.g., Wilson 1976, 1980a; Seid and Traniello 2006; Camargo et al. 2007; Lillico-Ouachour and Abouheif, 2017). Our ethogram data show that performance of major behavioral categories and the composition of behavioral repertoires are important parameters distinguishing labor by worker subcastes. Increased interaction of minor workers with refuse dumps and dead nestmates is a novel finding among leaf-cutters. The types of tasks executed by different subcastes of *A. subterraneus* closely resemble the social organization and division of labor observed in *Atta* colonies (Wilson 1976, 1980a) and other monomorphic or weakly polymorphic fungus-farming ants (Ronque 2018). This suggests that alongside “leaf-cutter agriculture,” a highly conserved set of worker behaviors persist since the origin of the leaf-cutting lineage, or even of the whole lineage of fungus-farming ants (Mehdiabadi and Schultz 2009). Further behavioral studies comparing the repertoires of *Acromyrmex* to *Trachymyrmex* and *Sericomyrmex* species (non-leaf-cutters that occasionally cut leaves; Seal and Tschinkel 2008; Ronque et al. 2019), should clarify what kinds of behavioral changes (in frequency or composition) emerged due to the leaf-cutting habit, and which behaviors persisted through evolutionary conservatism. Our work highlights the importance of detailed analyses of the composition of behavioral repertoires in polymorphic fungus-farming ants to better understand their social organization, and the mechanisms mediating division of labor among worker subcastes in the Attina.

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## Compliance with ethical standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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