Novel mandibular gland volatiles from Apterostigma ants

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ABSTRACT

The fungus-farming ants are a well-studied evolutionary radiation within the subfamily Myrmicinae that associate with a web of symbionts that span kingdoms. Members of the Apterostigma pilosum species group cultivate unique basidiomycete fungi belonging to the coral-mushroom family Pterulaceae, a family of fungi that is distantly related to the Agaricaceae (Leucoagaricus and Leucocoprinus) fungi grown by most fungus-farmers including other members in the genus Apterostigma (A. auriculatum group and A. megacephala). A chemical analysis using gas chromatography—mass spectroscopy of the mandibular gland volatiles of two species — A. dentigerum and A. manni — revealed the presence of an extraordinary diversity of natural products. Many of these compounds are new to Arthropoda, such as a homologous series of 3-methyl-2-alkanones, 2-methyl-2-alkenals, and 1-phenyl-2-propanone in A. dentigerum and 1-phenyl-2-propanol in A. manni. These results identify a remarkable divergence of compounds across the fungus-growing ants and other members in Myrmicinae. Functions of these natural products are proposed and discussed.

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1. Introduction

The fungus-farming ants (Hymenoptera: Formicidae: Tribe Attini: Atta genus-group, referred to in the narrow sense as "attine" hereafter) (Ward et al., 2015) have been growing fungi for ca. 55—65 million years (Ješovnik et al., 2016; Nygaard et al., 2016; Schultz and Brady, 2008; Ward et al., 2015) and have diverged into distinct lineages. To date, there are more than 250 New World species across 16 genera (Bolton, 2016; Klingenberg and Brandão, 2009; Sosa-Calvo et al., 2015, 2013). The majority of attine species cultivate a narrow range of parasol mushrooms in the tribe Leucocoprineae (Basidiomycota: Agaricaceae) but the species in the genus Apterostigma Mayr are unique in this respect (Schultz et al., 2015). This lineage of ants has a remarkably diverse fungal preference, from Agaricaceae (parasol) to Pterulaceae (coral) fungi.

Apterostigma currently consists of 45 extant and two extinct species (Lattke, 1999, 1997; Schultz, 2007) and presents a wide

geographic distribution in the New World tropics, from southern Mexico to northern Argentina (Lattke, 1997). Aside from extinct species known from Dominican amber, the genus is not found around the Caribbean (Fernandez and Sendoya, 2004; Lattke, 1997; Sosa-Calvo et al., 2015; Wilson, 1988). Studies on Apterostigma species have ranged widely, from investigating their large genome size (Tsutsui et al., 2008), to identifying new antibiotics (Carr et al., 2012; Freinkman et al., 2009; Oh et al., 2009; Van Arnam et al., 2016) to natural history descriptions (Pitts-Singer and Espelie, 2007). In general, Apterostigma workers are monomorphic and colonies tend to be small (<100 workers) and usually monogynous (Black, 1987; Forsyth, 1981; Pitts-Singer and Espelie, 2007; Weber, 1972). Most species of Apterostigma are known to nest in a variety of places, including in rotten logs, in cavities in the soil, under stones, or in the leaf litter, at the base and in between leaves of bromeliads, and on the undersides of palm leaves (Black, 1987; Forsyth, 1981; Lattke, 1997; Mehdiabadi and Schultz, 2009).

In the Panama Canal region, A. dentigerum Wheeler nest in exposed, polydomous hanging gardens in creek embankments, whereas a single colony of A. manni Weber was collected from six underground chambers in the forest (Adams unpublished). Both

ant species grow their fungal cultivars in dense sponge-like mats, typical of fungus-farming ant species; however, veil-like fungal structures are also seen in both species. The veil is used to cover the exposed areas of the fungus garden in *A. dentigerum,* while it was found lining the tunnels between chambers in *A. manni. Apterostigma* species that grow exposed hanging gardens are able to protect their cultivars from microbial pathogens that are constantly bombarding the outer surface of the fungal garden. It has been suggested that this protection is achieved due to the association with co-evolved antibiotic-producing bacterial symbionts grown on the exoskeleton of the ants (Caldera and Currie, 2012), but may also be augmented by ant derived chemical compounds (Poulsen et al., 2002; Vieira et al., 2012).

Studying the function of ant-derived compounds provides unique insight into the biology of the ants. The mandibular glands of ants have been found to be the source of numerous volatile organic compounds strongly associated with behavior (Hernández et al., 1999). Through extensive exploration of the communication systems in the higher attines — such as the leaf cutting genera *Atta* and *Acromyrmex* — it has been discovered that many of these natural products are common among other fungus-farming ants as well (Adams unpublished). Relatively small and simple aliphatic ketones such as 4-methyl-3-heptanone, 2-heptanone, and 3-octanone have been demonstrated to act as alarm pheromones in many species (de Lima Mendonça et al., 2009; Jackson and Morgan, 1993; Moser et al., 1968). However, ant-derived compounds are not only used in communication but also help to transform the ants' living surroundings by killing microbes in the nest soil and suppressing entomopathogens attacking ant brood and workers (Fernández-Marín et al., 2006; Vander Meer, 2012). Ants depend upon a suite of compounds that perform various functions. For example, the metapleural glands of many species have been found to produce antimicrobial compounds used to control and eliminate the presence of fungal pathogens (Bot et al., 2002; de Lima Mendonça et al., 2009; Fernández-Marín et al., 2015). However, except for an investigation into *Apterostigma* metapleural glands by

Vieira et al., 2012, the chemical ecology of this genus and other paleoattines has largely been ignored.

In this study, we focus on the mandibular gland compounds of two species in the *Apterostigma pilosum* species group, *A. dentigerum* and *A. manni.* Although *A. dentigerum* and *A. manni* belong to the same species group and tend coral fungal cultivars—albeit from different clades (G2 and G4) (Dentinger et al., 2009; Villesen et al., 2004)—they produce distinct and different natural products.

2. Materials and methods

2.1. Collection and identification

Colonies were collected live from two sites in Parque Soberanía, Panama, on Pipeline Road, Km 6 (9.1645−79.74557) and Rio La Seda (9.1562−79.73447), *A. manni* and *A. dentigerum* respectively (see Table 1 for collection codes). Samples of workers were taken from the lab colonies no more than two months following their collection date. Specimens were identified to species by using Lattke's key to species (1997) and by direct comparison with specimens deposited in the insect collection of the Smithsonian Institution National Museum of Natural History, in Washington DC.

2.2. Dissections

Methanol extracts of pooled samples of various body sections and glands (ant heads, thoraxes, abdomens, and whole ants; mandibular glands, propharyngeal glands, and postpharyngeal glands) were analyzed with gas chromatography−mass spectrometry (GC−MS), with the goal of identifying the mandibular gland compounds and determining their glandular origin.

Before dissection, ants were anesthetized on ice and rinsed in ethanol and methanol for five seconds to remove exoskeleton compounds. Specimens were either trisected or the head glands dissected. Dissections of mandibular, propharyngeal and

Table 1
Specimens used in the chemical analysis.

Species	Colony ID	Collection code	Body part (# ants)	Compounds present?
Apterostigma dentigerum	RMMA150518-01	RMMA18-01-H	Head (19)	Yes
		RMMA18-01-T/A	Thorax and abdomen (20)	No
	RMMA150525-10	RMMA25-H	Head (10)	Yes
		RMMA25-T	Thorax (10)	No
		RMMA25-A	Abdomen (10)	No
		RMMA25-POST	Postpharyngeal gland (10)	No
		RMMA25-PRO	Propharyngeal gland (10)	No
		RMMA25-MAN	Mandibular gland (10)	Yes
	JS150511-03	JS11-03-H	Head (11)	Yes
		JS11-03-T/A	Thorax and abdomen (11)	No
		JS11-03-POST	Postpharyngeal gland (10)	No
		JS11-03-PRO	Propharyngeal gland (10)	No
		JS11-03-MAN	Mandibular gland (10)	Yes
	JS150511-01	JS11-01-WA	Whole ant (10)	Yes
		JS11-01-POST	Postpharyngeal gland (10)	No
		JS11-01-PRO	Propharyngeal gland (10)	No
		JS11-01-MAN	Mandibular gland (10)	Yes
	JS150509-01	JS09-01-H	Head (8)	Yes
		JS09-01-T/A	Thorax and abdomen (8)	No
Apterostigma manni	RMMA150523-05	RMMA23-05-WA	Whole ant (5)	Yes
		RMMA23-05-H9	Heads (9)	Yes
		RMMA23-05-H5	Heads (5)	Yes
		RMMA23-05-T/A	Thorax and abdomen (9)	No
		RMMA23-05-T	Thorax (5)	No
		RMMA23-05-A	Abdomen (5)	No
		RMMA23-05-POST	Postpharyngeal gland (17)	No
		RMMA23-05-PRO	Propharyngeal gland (8)	No
		RMMA23-05-MAN	Mandibular gland (17)	Yes

postpharyngeal glands were performed under a stereoscopic microscope in distilled water on glass slides, and washed with ethanol and methanol. Glands were dissected with forceps rinsed with solvents (ethanol and methanol), propharyngeal and post-pharyngeal glands were separated by cutting with sterile surgical blades. After removal from the head, glands were washed in a fresh portion of water. All samples were collected in an equal quantity of 20 μl methanol. GC–MS analyses were performed on the methanol extracts of 8–19 ant heads as well as 10–17 excised glands (see Table 1 for complete list of all analyzed ant parts).

2.3. Chemical analysis

Gas chromatography–mass spectrometry was performed at Virginia Military Institute in the EI mode using a Shimadzu QP 2010 GC/MS equipped with a RTX-5, 30 m × 0.25 mm i.d., column (Restek Corporation, 110 Benner Circle, Bellefonte, PA 16823). The instrument was programmed from 60 to 250 °C at 10 °C/min and held at the upper temperature for 30 min.

3. Results

The objective of this study was to identify the mandibular gland volatiles of two closely related *Apterostigma* species. The chromatograms illustrate the retention times and relative abundance of the compounds of interest, highlighting the striking difference between *A. dentigerum* and *A. manni* (Fig. 1); peaks not marked with a number are due to chromatographic background noise and do not represent ant-derived compounds. Samples that included only the thorax and/or abdomen did not have the volatiles listed in Fig. 1, nor did the pro- and postpharyngeal gland samples (Table 1).

3.1. Apterostigma dentigerum

The methanol extracts of all samples of the mandibular glands of *A. dentigerum* revealed the presence of numerous volatile compounds (Fig. 1). The mass spectra of three of the components, peaks **5**, **7**, and **9**, showed fragments at M-15 and $m/z = 72$ suggestive of 3-methyl-2-alkanones, and matched the spectra provided for 3-methyl-2-octanone (**5**), 3-methyl-2-nonanone (**7**) and 3-methyl-2-decanone (**9**) (Burger et al., 2008). Additionally, the structures of peak **5**, **7**, and **9** were confirmed by direct comparison with synthetic samples (Radulović et al., 2014).

Peaks **1**, **4**, **6a**, **10**, and **11** seemed to be a homologous series of unsaturated compounds ($M^+ = 112$, 126, 140, 154, and 168 respectively), having a characteristic fragment at $m/z = 97$ in their
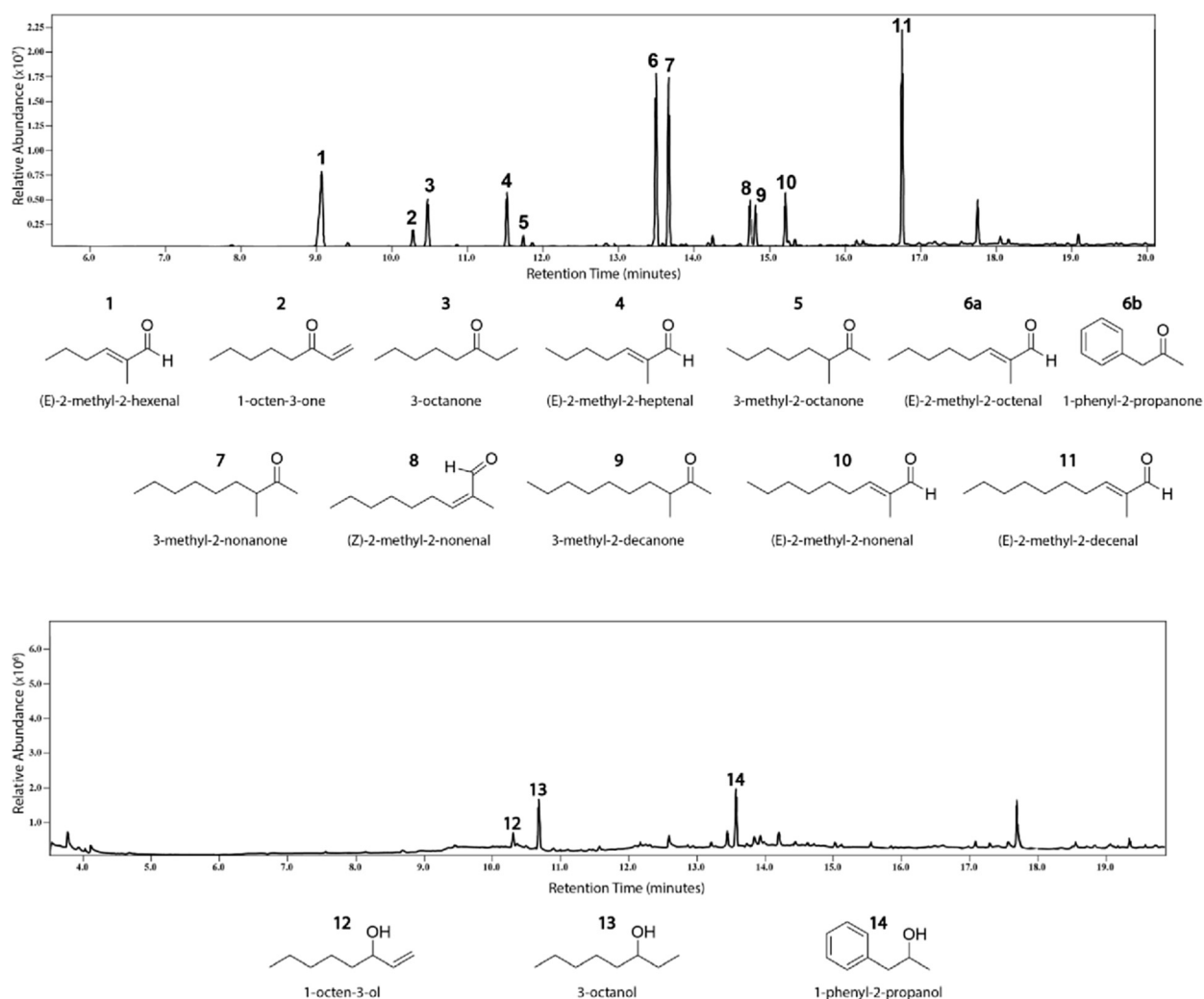

Fig. 1. GC/MS chromatograms of the mandibular gland volatiles from *A. dentigerum* and *A. manni*, respectively. Peaks not marked with a number are due to chromatographic background noise and do not represent ant-derived compounds.

mass spectra. Following microhydrogenation of the mixture over a small amount PtO₂, peak **11** appeared to have been reduced to 2-methyl-1-decanol. The mass spectrum of peak **11** matched the spectrum obtained for (E)-2-methyl-2-decenal (**11**) (Petroski et al., 2011), while the mass spectrum of peak **4** matched that reported for (E)-2-methyl-2-heptenal (**4**) (US Department of Commerce NIST, 2016). The Horner-Emmons condensation of butanal, hexanal, heptanal or octanal with triethyl 2-phosponopropionate followed by lithium aluminum hydride reduction and subsequent pyridinum chlorochromate oxidation provided synthetic samples of (E)-2-methyl-2-hexenal (**1**), (E)-2-methyl-2-octenal (**6a**), (E)-2-methyl-2-nonenal (**10**), and (E)-2-methyl-2-decenal (**11**) respectively (Jones et al., 1977; Naef and Jaquier, 2006). Additionally, the gas chromatographic retention time as well as the mass spectra of peak **8** appeared to indicate (Z)-2-methyl-2-nonenal (**8**), and isomer of compound **10**, although further investigation is necessary to confirm the identity of this compound. The mass spectra and gas chromatographic retention times of synthetic samples of (E)-2-methyl-2-alkenals were identical to those of peaks **1**, **4**, **6a**, **10**, and **11** respectively by direct comparison.

Finally, the mass spectra of peaks **2** and **3** matched that reported for 1-octen-3-one (**2**) and 3-octanone (**3**), while treatment of the extract with a small amount of ethereal diazomethane revealed the presence of octanoic, nonanoic, and decanoic acid as their methyl esters (US Department of Commerce NIST, 2016). Careful examination of the region between peaks **6** and **7** showed the presence of ions at $m/z = 91$ and $m/z = 134$ congruent with one another and representing the presence of 1-phenyl-2-propanone (**6b**) (US Department of Commerce NIST, 2016).

3.2. Apterostigma manni

The methanol extracts of the mandibular glands of *A. manni* revealed a relatively simple mixture without the complexity seen in *A. dentigerum* (Table 1). Examination of the mass spectra revealed the presence of the 1-octen-3-ol (**12**) and 3-octanol (**13**) along with 1-phenyl-2-propanol (**14**) (US Department of Commerce NIST, 2016).

4. Discussion

Our chemical analyses highlight many differences between the mandibular gland chemistry of *A. dentigerum* and *A. manni*, prompting further questions concerning the function of these compounds. The leaf cutting ant genera (i.e., *Atta* and *Acromyrmex*) share many mandibular gland volatiles across species, suggesting a conservation of compounds between the two sister lineages (de Lima Mendonça et al., 2009, and references therein). For this reason, we expected some degree of conservation in mandibular compounds between these *Apterostigma* species as well. Instead, we found that the mandibular glands of *A. dentigerum* contained a series of methyl-branched aldehydes and ketones that were not detected in *A. manni*. However, three of the 14 compounds found in *A. dentigerum* (1-octen-3-one (**2**), 3-octanone (**3**) and 1-phenyl-2-propanone (**6b**)) appeared in their reduced forms and as the only detectable volatile compounds in *A. manni* (1-octen-3-ol (**12**), 3-octanol (**13**), and 1-phenyl-2-propanol (**14**)). This is the first time that either compound **6b** or **14** have been reported from any member of the phylum Arthropoda. The presence of 3-oxygenated octanes in both species is not surprising since many members of the subfamily Myrmicinae produce such compounds as major components of their mandibular glands (Attygalle and Morgan, 1984; Cammaerts et al., 1983; Crewe et al., 1972; Crewe and Blum, 1972; de Lima Mendonça et al., 2009; Jackson and Morgan, 1993).

Mandibular gland secretions are known to play a role in communication (Blum, 1969). 3-octanone (**3**) functions as an alarm pheromone in the leaf cutting ant species, *Atta texana* (Moser et al., 1968), *Acromyrmex octospinosus*, and *Acromyrmex landolti* (Sainz-Borgo et al., 2013) and have been identified in a number of other higher attine species (*Trachymyrmex cornetzi, Trachymyrmex septentrionalis, Trachymyrmex urichii,* and *Sericomyrmex amabilis*) (Adams et al., 2012; Crewe et al., 1972). 3-octanol (**13**) has been found in the mandibular glands of forty-two species of *Myrmica* and *Crematogaster* (Cammaerts et al., 1985; Longhurst et al., 1980) and is also used in colony communication and serves as an attractant in *Myrmica* ants (Cammaerts et al., 1985). Compounds **3** and **13** appear to be broadly conserved alarm pheromones across the myrmicine ants (Cammaerts et al., 1983; Crewe et al., 1972; de Lima Mendonça et al., 2009) including a number of attine genera (Adams et al., 2012; Blum, 1981). Additionally, 1-octen-3-one (**2**) and 1-octen-3-ol (**12**) have not yet been reported from ants but are recognized as components of fungal odor (Davis et al., 2013; Steiner et al., 2007).

A homologous series of 3-methyl-2-alkanones (3-methyl-2-octanone (**5**), and 3-methyl-2-nonanone (**7**) 3-methyl-2-decanone (**9**)), was identified from the mandibular gland extracts of *A. dentigerum* (Table 1). Volatiles (**9**) and (**5**) have not been reported from arthropods and all three 3-methyl-2-alkanones have been found in Bengal tiger urine (Burger et al., 2008). In *A. dentigerum,* 3-methyl-2-nonanone (**7**) was a major component of the mandibular gland volatiles (Table 2) and has previously been reported from the same gland of distantly related *Diacamma* (Ponerinae) ant species (Morgan et al., 1999). Although their function remains to be elucidated, these compounds may serve in a communicative capacity in *A. dentigerum,* given that low molecular weight aliphatic alcohols and ketones such as these have been found to act as semiochemicals in many ant species (Brown et al., 1970; Jackson and Morgan, 1993; Moser et al., 1968).

Independent of communicative functions, some fungus-farming ant derived volatiles act as antimicrobials (de Lima Mendonça et al., 2009; Knapp et al., 1994; Rodrigues et al., 2008). We report a series of homologous 2-methyl-2-alkenals from the mandibular glands of *A. dentigerum* (Table 1, **1**, **4**, **6a**, **8**, **10**, **11**). These volatile compounds have never before been detected in the phylum Arthropoda, but are found in lemon peel extract (Naef and Jaquier, 2006). However, related compounds citral and geronial serve as antifungal and antibacterial agents in leaf cutting

Table 2
Relative amount of mandibular gland compounds.

Peak #	Compound	A. dentigerum	A. manni
1	(E)-2-methyl-2-hexenal	(0.64)	-
2	1-octen-3-one	(0.09)	-
3	3-octanone	(0.26)	–
4	(E)-2-methyl-2-heptenal	(0.26)	–
5	3-methyl-2-octanone	(0.05)	–
6a	(E)-2-methyl-2-octenal	(0.79)	–
6b	1-phenyl-2-propanone	(*)	–
7	3-methyl-2-nonanone	(0.76)	–
8	(Z)-2-methyl-2-nonenal	(0.20)	–
9	3-methyl-2-decanone	(0.18)	–
10	(E)-2-methyl-2-nonenal	(0.22)	-
11	(E)-2-methyl-2-decenal	(1)	-
12	1-octen-3-ol	–	(0.56)
13	3-octanol	–	(1)
14	1-phenyl-2-propanol	–	(0.33)
15	Octanoic acid	(*)	–
16	Nonanoic acid	(*)	–
17	Decanoic acid	(*)	–

Asterisk (*) indicates only a trace amount was found and hyphen (−) indicates absence.

ant species (de Lima Mendonça et al., 2009; Knapp et al., 1994). These effective antimicrobials (Onawunmi, 1989) may provide insight into the function of the 2-methyl-2-alkenals, given their structural similarity. Citral and geronial − both structural isomers of compound **11** − possess a conjugated carbonyl functional group that is highly reactive and volatile in nature; thus, the ant-derived 2-methyl-2-alkenals may prove to be a part of the *A. dentigerum* chemical defenses. Disk diffusion assays suggests both (E)-2-methyl-2-octenal (**6a**) and (E)-2-methyl-2-decenal (**11**) are powerful growth-inhibiting agents effective against both Gram-positive and Gram-negative bacterial strains (Hogan unpublished), although further analysis is necessary to determine the efficacy of these compounds.

Our chemical analyses also revealed the presence of octanoic, nonanoic, and decanoic acids (Table 1, **15**, **16**, **17**) from the mandibular glands of *A. dentigerum*. The strong antibacterial and antifungal activity of medium chain carboxylic acids of this type has previously been reported by Koidsumi (1957), and have been found to be a major component of the metapleural glands of *Acromyrmex* leaf cutting ants (Bot et al., 2002). Recent investigations have demonstrated the effectiveness of hexanoic and octanoic acid in the inhibition of various bacterial and fungal strains (de Lima Mendonça et al., 2009), leading to the hypothesis that compounds **15**, **16**, and **17** may serve a similar function in *A. dentigerum*.

Within the genus *Apterostigma*, the *A. pilosum* species group cultivates a basidiomycete fungus belonging to the coral-mushroom family Pterulaceae (G2 and G4 clades), while others cultivate lepiotaceous (Agaricaceae parasol mushroom) cultivars, as typical in many other fungus-farming ant species (Schultz and Brady, 2008). Most recently, it has been discovered that *Apterostigma megacephala* Lattke, a relictual species of fungus-farming ant, is the only non-leafcutter ant that cultivates the highly derived *Leucoagaricus gongylophorus* fungus, known to be only associated with the leaf cutting ant genera *Acromyrmex* and *Atta* (Schultz et al., 2015). The flexible cultivar acquisition behavior among *Apterostigma* species may enable a colony to re-acquire cultivars after garden loss or purge inferior cultivars that are locally maladapted (Kellner et al., 2013). Novel ant−fungus combinations adapted to varying environmental conditions likely provide evolutionary advantages (Himler et al., 2009), and the unusual mandibular gland chemistry of *A. dentigerum* may be related to the cultivation of the unique G2 pterulaceous fungi.

Additionally, compounds acting as generalized antimicrobials in *A. dentigerum* could have beneficial consequences, as the protection of the fungal garden and ant workers against microbial pathogens is of paramount importance to the colony's survival. *Apterostigma dentigerum* fosters an antibiotic-producing *Pseudonocardia* bacterium on its exoskeleton, a symbiotic relationship that aids the ants in the defense against *Escovopsis* fungal pathogens (Caldera and Currie, 2012; Currie et al., 1999; Gerardo et al., 2006; Mueller et al., 1998). However, this relationship may only protect the fungal garden from one or a few of many threats (Fernandez-Marin et al., 2009; Sen et al., 2009). The exposed hanging gardens of *A. dentigerum* are constantly challenged with numerous microbial pathogens (Chapela et al., 1994; Munkacsi et al., 2004; Villesen et al., 2004), therefore it would not be surprising if *A. dentigerum* mandibular gland compounds function as antimicrobials used to defend against a broad range of microbial pathogens.

5. Conclusions

This investigation into the chemical ecology of the *A. pilosum* species group has demonstrated unique characteristics that distinguish these species from the rest of the fungus-farming ants.

While the results of this study have revealed the presence of many new mandibular gland compounds, the functions of these natural products remain to be determined. Preliminary microbiological assays with the series of 2-methyl-2-alkenals from *A. dentigerum* suggest antimicrobial efficacy, and research in this area is forthcoming. The phylogenetic relationships within the *A. pilosum* species group, and other *Apterostigma* species, are currently being reconstructed, which will allow the examination of chemical trait evolution across the entire genus. In order to unravel the natural history and phylogeny of the fungus-farming ants, more research must be conducted to link their chemical ecology with their phylogenetic placement.

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