

Pygidial gland chemistry and potential alarm-recruitment function in column foraging, but not solitary, Nearctic *Messor* harvesting ants (Hymenoptera: Formicidae: Myrmicinae)



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ABSTRACT

We investigated the role of the pygidial gland on foraging behavior in two ecologically dominant column foraging Nearctic harvesting ants (*Messor pergandei* and *Messor andrei*). Using chemical analyses and behavioral tests, we show that n-tridecane is the major biologically active compound of pygidial gland secretions in both species, and that this chemical functions as a powerful alarm-recruitment pheromone. Another major compound of pygidial gland contents is benzaldehyde; this substance does not release behavioral reactions in *M. pergandei* workers but might function as a defensive secretion. Six solitary foraging Nearctic *Messor* and two column foraging Palearctic *Messor* species, did not have large pygidial gland reservoirs.

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1. Introduction

The typical ant worker is a walking battery of exocrine glands, developed to a degree well beyond that typical of nonsocial hymenopterans (Billen and Morgan, 1998; Hölldobler and Wilson, 1990). Exocrine glands vary greatly in form and distribution among ant subfamilies, and many of these glands have been implicated in production of semiochemicals or defensive secretions which are used in social communication (Hölldobler and Wilson, 1990, 2009; Morgan, 2009). Janet (1898) discovered the pygidial gland as a cluster of a few cells under the VIth abdominal tergite, with ducts leading to the intersegmental membrane between the VIth and VIIth tergites. After 80 years it was rediscovered as a well-developed organ in *Aphaenogaster* (= *Novomessor*) (Hölldobler et al., 1976), and it was subsequently shown to be widespread among other genera of Myrmicinae (Hölldobler and Engel, 1978; Kugler, 1978). Once defined anatomically in the Myrmicinae, the pygidial gland was also located in the subfamilies Ponerinae, Ectatominae, Myrmecinae, Dorylinae, Ecitoninae, Pseudomyrmecinae, Leptanillinae, Aneuretinae and Dolichoderinae (Hölldobler

and Engel, 1978; Hölldobler et al., 1989). Originally researchers had diagnosed Dolichoderinae in part by possession of the supposedly unique “anal gland” (Pavan and Ronchetti, 1955), but anatomical studies determined the homology of this “anal gland” with the pygidial gland in other ant subfamilies (Hölldobler and Engel, 1978; Billen, 1986). Species of Formicinae lack this gland, except in the slave raiding genus *Polyergus*, where it appears as a secondary evolutionary development (Hölldobler, 1984).

The pygidial gland is widespread and functionally diverse in the Ponerinae (Fanfani and Dazzini Valcurone, 1986; Hölldobler and Engel, 1978; Jessen et al., 1979). In several species the secretions elicit either recruitment or sexual attraction (see Hölldobler and Wilson, 1990). In the Dolichoderinae, pygidial gland secretions have a defensive function, yet in one species, *Tapinoma simrothi*, it has been reported that pygidial gland secretions serve as trail pheromone (Simon and Hefetz, 1991).

Very little is known about function of pygidial gland secretions in myrmecine ants. Workers of *Aphaenogaster* (= *Novomessor*) *albisetosus* and *Aphaenogaster* (= *Novomessor*) *cockerelli* release strong-smelling components that evoke a form of panic alarm, which evidently serves to organize swift evacuations during the approach of army ants (Hölldobler, unpublished; McDonald and Topoff, 1986; Smith and Haight, 2008). In *Pheidole biconstricta*, minor workers produce large quantities of a secretion from hypertrophied pygidial glands that is used in both chemical defense and

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aggressive alarm (Kugler, 1979). Subsequently, Davidson et al. (2005) evaluated the chemistry of whole gasters of methanol preserved specimens of *P. biconstricta* and found iridodials and actinidine as primary components. In *Pheidole embolopyx*, major workers discharge alarm pheromones from the pygidial gland (Wilson and Hölldobler, 1985). Morgan et al. (2006) describe large pygidial glands in *Ocymyrmex laticeps*, which primarily contain indole-3-ethanol, but nothing is known about its behavioral function. This paper presents the first chemical-behavioral analysis of pygidial gland secretions in myrmicine ants. We checked eleven Nearctic *Messor* species, and two column foraging Palearctic *Messor* species, for the presence of the pygidial gland. We investigated the role of the pygidial gland components during initiation of column foraging in two New World *Messor* species (formerly *Veromessor*): *Messor pergandei* (Mayr) and *Messor andrei* (Mayr). Both species are ecologically dominant harvester ants whose colonies conduct group foraging during which thousands of foragers travel in huge columns to harvesting sites (see Plowes et al., 2013 for a review of foraging behavior and ecology in New World and Old World *Messor* species).

2. Methods

2.1. Laboratory protocol for *M. pergandei* and *M. andrei*

Foragers of *M. pergandei* were collected from nests at South Mountain Park, Phoenix, Maricopa County, AZ, while those of *M. andrei* were collected at Table Mountain Preserve, Auberry, Fresno County, CA, and Pine Valley, San Diego County, CA. Laboratory experiments, dissections, and morphological studies, were performed at Arizona State University, Tempe, AZ. We collected 2000–3000 worker ants from at least 10 different field colonies per species, and divided each collected colony into 2–3 test groups. Each test groups consisted of 500–1000 workers, which were placed in large Plexiglas arenas (50 cm × 100 cm) with a 3 cm layer of plaster. Test groups were used for several different behavioral tests, with breaks of 24–48 h between trials. Nest spaces (either 10 cm × 15 cm, or 15 cm × 20 cm) in the plaster had glass lids, and were covered with red acetate film. Test groups were fed Bhatkar diet, sugar water, and Kentucky ryegrass seeds *ad libitum*. The plaster floor and nests were kept moist. Arenas were maintained in a room with 12 h light/dark cycle at approximately 26 °C. The workers moved into nest spaces. The majority of workers remained in the nest, while small numbers (100+) ventured into the arena to forage for food and water. Worker behavior was similar to that observed in the field, with workers forming columns and foraging during the early morning and returning to the nest space at night.

2.2. Morphological studies

We prepared six Nearctic *Messor* species (*M. andrei*, *M. julianus*, *M. lariversi*, *M. pergandei*, *M. smithi*, *M. stoddardi*) for examination by SEM. Tergites and abdomens from freshly frozen or ethanol preserved ants were dissected under water, rinsed, and placed on filter paper to air dry for 24 h. Specimens were then mounted on brass stubs with carbon adhesive tabs and placed in a dessicator for 72 h. Specimens were prepared for SEM viewing by sputter coating with approximately 15–20 nm of gold/palladium. They were viewed on a JEOL JSM-6300 SEM fitted with an IXRF digital imaging system. Histological preparations of *M. pergandei*, and *M. barbarus* included fixing ants in alcoholic Bouin or Carnoy, imbedding specimens in methyl methacrylate, then sectioning by microtome, followed by Azan staining (for further details, see Hölldobler et al., 1976; Hölldobler and Engel, 1978). Fresh specimens of *M. andrei*, *M. pergandei*, *M. smithi*, *M. barbarus* and *M. lusitanicus* were

dissected under water to check for the presence of pygidial gland reservoirs.

2.3. Chemical analysis

Glands were excised from freshly frozen workers of *M. pergandei* and *M. andrei*. Pygidial glands still attached to tergites were mounted on shards of glass and placed inside borosilicate capillary tubes which were then flame sealed for shipment from Arizona to New Jersey. Chemical analyses were performed at the Stevens Institute of Technology, New Jersey. Capillaries were crushed and gland contents extracted with dichloromethane (20 µL). One microliter aliquots of extract were subjected to GC–MS analysis on a Hewlett–Packard 5971 GC–MS instrument fitted with an HP-5-MS coated (0.25 µm) fused silica capillary column (30 m × 0.25 mm). Oven temperature was held at 50 °C for 3 min, then increased at 8 °C/min to 250 °C and maintained at 250 °C for 10 min. Helium was used as the carrier gas (1 mL/min). Samples were introduced by splitless injection (injection port temperature 280 °C). Positive-ion electron-ionization (70 eV) mass spectra were acquired at a rate of one spectrum per second. Compounds were identified by spectral interpretation and comparison with authentic spectra in Wiley Registry of Mass Spectral Data (8th Edition).

2.4. Behavioral analysis

Experimental worker groups were tested in the laboratory between 6 and 10 am, during the daily period of peak foraging activity observed in the field. Behavioral tests were run using crushed pygidial glands, hexane extract of the pygidial gland secretions, or synthesized chemicals (n-tridecane (Sigma Aldrich), pentadecane (Sigma Aldrich), benzaldehyde (Sigma Aldrich), benzoic acid (Sigma Aldrich)) in hexane solvent. In pilot tests with artificial trails drawn with pygidial gland secretions, we determined that pygidial gland secretions did not elicit sustained trail following, but the ants were attracted to point sources of pygidial gland secretions (Video 1).

To analyze the following response of ants to pygidial gland secretions, we designed the following experimental procedures. First we presented the tips of hardwood applicator sticks (on which a single freshly dissected pygidial gland had been crushed) close to the nest entrance. Alternatively, we used hexane extracts of one gland equivalent of pygidial gland secretions (25 glands placed in 100 µL of hexane, ~4 µL of this solution was used), applied with a microsyringe onto a small piece of filter paper. The filter paper was held with forceps, and after 10 s (to allow the hexane to evaporate) was held at the nest entrance, approximately 0.5–1 cm above the arena floor. We waited 20 s, then counted all ants in a 6 cm × 6 cm square around the applicator stick or filter paper. We then moved the applicator stick or filter paper slowly 20–30 cm to a second square of 6 cm × 6 cm, not touching the arena floor, and took a count within this second square. Controls were run in the same manner, but with 3 µL of pure hexane. We followed the same procedure when testing whether *M. andrei* and *M. pergandei* demonstrated behavioral responses to each other's pygidial glands.

To test putative pygidial gland pheromones, we made hexane solutions of n-tridecane, pentadecane, benzoic acid and benzaldehyde. In pilot tests, we tested solutions ranging in concentration from 50 ppm to 0.1 ppm. The behavioral responses that most closely mimicked natural following behavior were found in the 5 ppm range, so this was the concentration used for standardized tests reported in the results. We followed the same procedure as before, except both test solutions (3 µL of 5 ppm hexane solution) and hexane controls were offered on a hardwood applicator.

3. Results

Sagittal sections through the gaster of *M. pergandei* workers revealed a well-developed pygidial gland, which consisted of paired large invaginations of the intersegmental membrane between the VIth and VIIth abdominal tergites (Fig. 1), and paired large clusters of glandular cells; each cell was connected by a duct to the intersegmental membrane that forms the reservoir sacks (Hölldobler et al., 1976; Hölldobler and Engel, 1978). The pygidial gland is associated with a conspicuous paired structure on the cuticle surface of the VIIth tergite (Fig. 2). Although we did not prepare histological sections of *M. andrei* workers, dissections of freshly killed ants revealed a large pygidial gland similar to that of *M. pergandei* workers, also associated with a distinct cuticle structure on the surface of the VIIth tergite (Fig. 3). We also investigated the pygidial glands of *M. julianus*, the third column foraging species in North America. Although we had only specimens preserved in ethanol, we could detect a distinct pygidial gland and the associated cuticle structure. On the other hand, in the six solitary foraging North American *Messor* species, we were unable to detect pronounced pygidial gland reservoirs or associated cuticle structures (Table 1). Similarly we could not detect pronounced pygidial glands in workers of the European species *M. barbarus* or *M. lusitanicus*.

The GC–MS analysis of pygidial gland secretions of *M. andrei* workers revealed the presence of several volatile substances (Fig. 4a). Their mass spectra and retention time matched those of (1) n-tridecane, (2) pentadecane, (3) 2,4-tetradecadienal, (4) hexadecanoic acid, (5) a mixture of 9,12-octadecadienoic acid, 9-octadecenoic acid, octadecanoic acid, and (6) pentacosane (listed in order of retention time). The blend of pygidial gland secretions of *M. pergandei* workers was distinctly different (Fig. 4b). A major component is (1) benzaldehyde, in addition we detected (2) benzoic acid, (3) n-tridecane, (4) mandelonitrile, (5) pentadecane, (6) 3-methylhexadecane, (7) hexadecanoic acid, and (8) a mixture of 9,12-octadecadienoic acid, 9-octadecenoic acid, octadecanoic acid (listed in order of retention time). Four compounds were found only in *M. pergandei* pygidial glands: benzaldehyde, benzoic acid, mandelonitrile, and 3-methylhexadecane. Four compounds (n-tridecane, pentadecane, hexadecanoic acid, octadecadienoic acid) were shared between both species. Only two compounds, 2,4-tetradecadienal and pentacosane, were unique to *M. andrei*.

Behavioral assays with crushed pygidial glands or hexane extracts of glandular secretions demonstrated that *Messor* workers of both species exhibited attraction to point sources of one gland equivalent of glandular contents (Video 1). Initially the attracted ants exhibited aggressive behavior such as brief gaping of

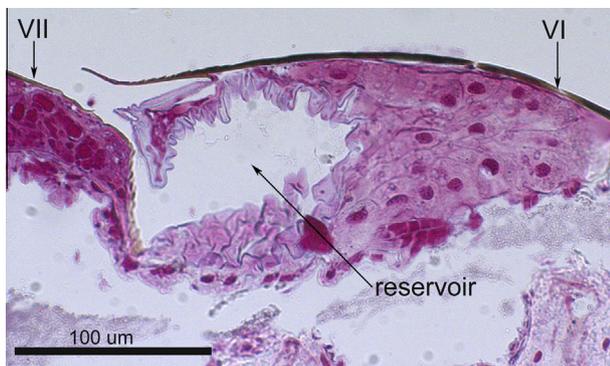


Fig. 1. Sagittal section of a *Messor pergandei* gaster, showing the opening of a pygidial gland reservoir between abdominal tergites VIth and VIIth. The gland reservoir (R) is an invagination of the intersegmental membrane. R – gland reservoir, GC – gland cells. (See Hölldobler et al., 1976 for histological technique.) Scale bar = 100 μm .

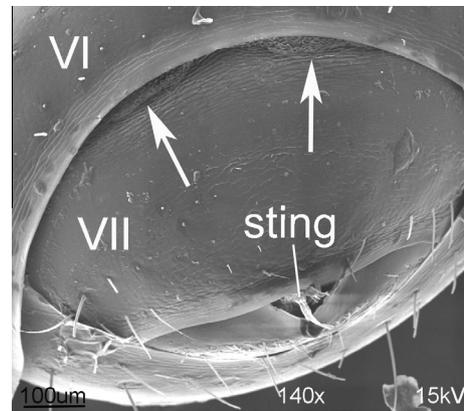


Fig. 2. Location of the openings to the pygidial glands (indicated by arrows) on the dorsum of *M. andrei* can be recognized by distinct changes in cuticle texture. Abdominal tergites VIth and VIIth are indicated. Scale bar = 100 μm .

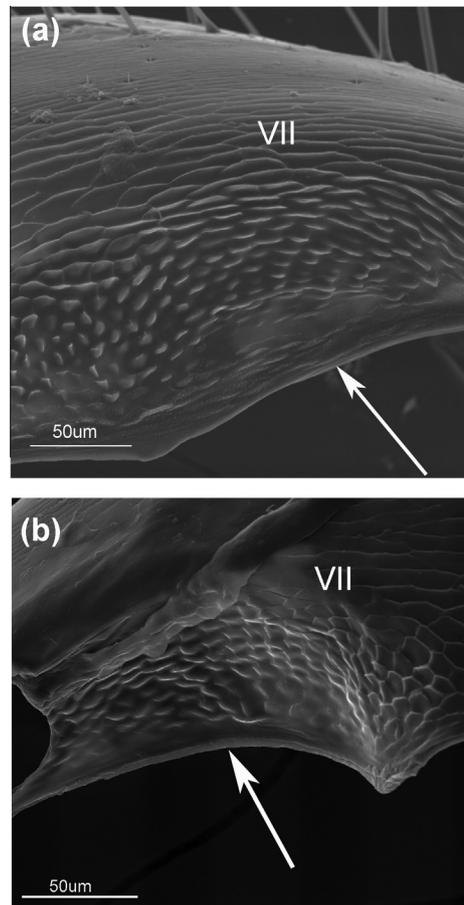


Fig. 3. (a) Texture of cuticle at gland opening in *M. andrei* (400 \times) and (b) *M. pergandei* (500 \times). The arrows point to the anterior edge of tergite VIIth, which attaches to the epithelial tissue of the pygidial reservoir. During normal ant walking posture, the first $\sim 100 \mu\text{m}$ of tergite VIIth is usually overlapped by tergite VIth. Scale bar = 50 μm .

mandibles and jerking movements. Such seemingly aggressive behavior waned after a few seconds; however the ants continued to roam around the point source. As documented in Fig. 5a for *M. pergandei*, significantly more ants emerged from the nest exit than in controls when presented with one ant equivalent of pygidial gland secretions (*T*-test, $t = -4.27$, $p < 0.005$, $n = 15$). About half of the ants induced to leave the nest or being attracted from near

Table 1

Nine Nearctic and two Palearctic species of *Messor* have been examined for the presence of pygidial gland reservoirs and associated tergal structures.

	Species (sample type)	Textured tergal cuticle	Presence of large pygidial gland reservoir
Nearctic, column foragers	<i>M. andrei</i> (fresh)	+	+
	<i>M. julianus</i> (ETOH)	+	+
	<i>M. pergandei</i> (fresh)	+	+
Nearctic, solitary foragers	<i>M. smithi</i> (fresh)	–	–
	<i>M. lariversi</i> (ETOH)	–	–
	<i>M. lobognathus</i> (ETOH)	–	–
	<i>M. stoddardi</i> (ETOH)	–	–
	<i>M. chamberlini</i> (ETOH)	–	–
	<i>M. chicoensis</i> (ETOH)	–	–
Palearctic, column foragers	<i>M. barbarus</i> (fresh)	–	–
	<i>M. lusitanicus</i> (fresh)	–	–

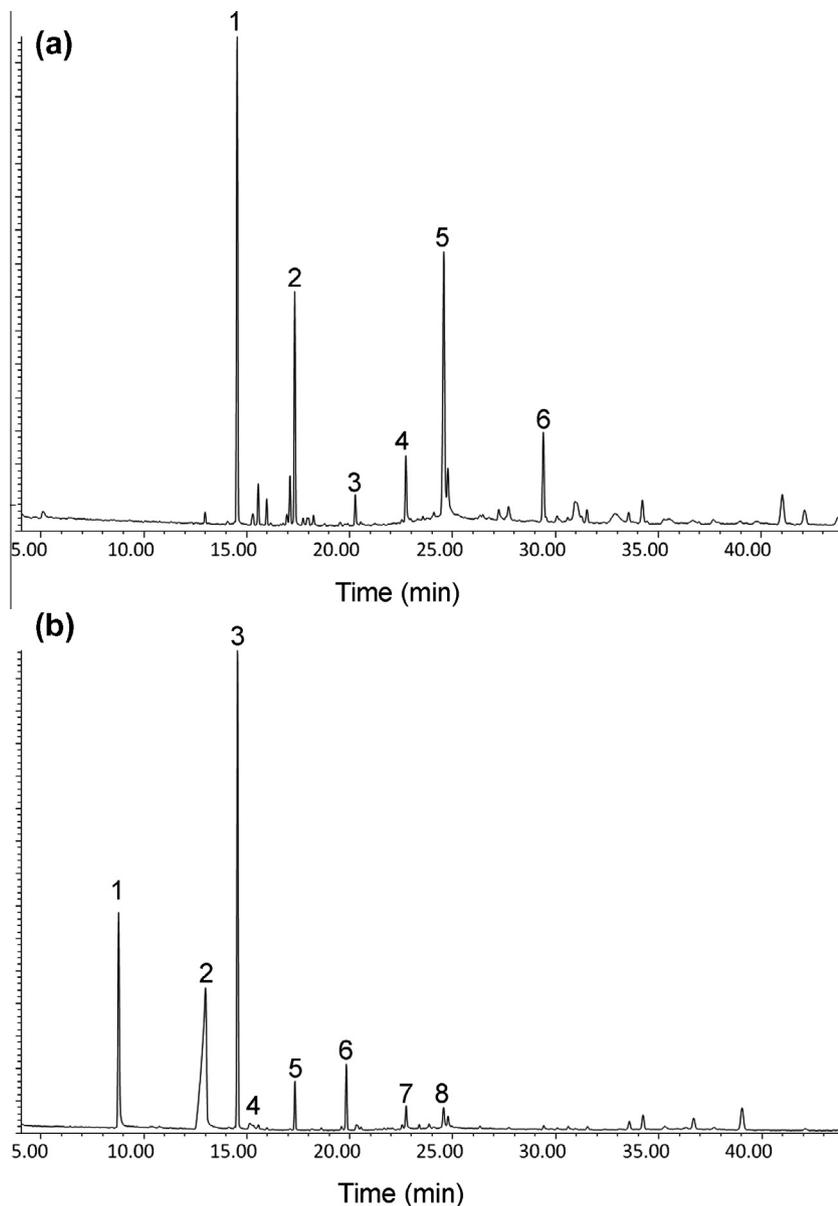


Fig. 4. (a) Total ion current CG mass chromatogram of the contents of three *M. andrei* pygidial glands. (1) n-tridecane, (2) pentadecane, (3) 2,4-tetradecadienal, (4) hexadecanoic acid, (5) mixture of 9,12-octadecadienoic acid, 9-octadecenoic acid, octadecanoic acid, (6) pentacosane. (b) Total ion current CG mass chromatogram of the contents of three *M. pergandei* pygidial glands. (1) benzaldehyde, (2) benzoic acid, (3) n-tridecane, (4) mandelonitrile, (5) pentadecane, (6) 3-methylhexadecane, (7) hexadecanoic acid, (8) mixture of 9,12-octadecadienoic acid, 9-octadecenoic acid and octadecanoic acid.

the nest exit followed the point source when it was moved slowly for a distance of about 30 cm. Similar results were obtained with *M. andrei* (Fig. 5b) (*T*-test, $t = -5.43$, $p < 0.005$, $n = 8$, unequal

variances). Although secretions from the pygidial gland elicited attraction in workers of both *Messor* species, they did not release trail following behavior, yet the ants followed a moving point

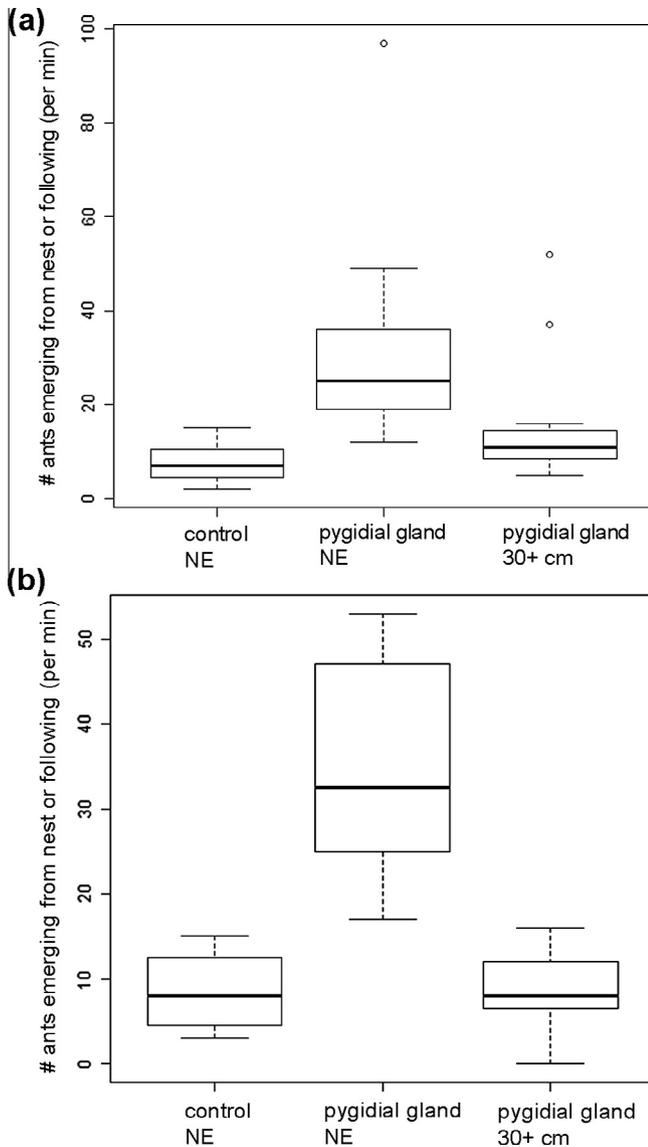


Fig. 5. Number of *M. pergandei* ($n = 15$) (a) or *M. andrei* ($n = 8$) (b) workers emerging from the nest entrance (NE) or following applicator stick for 30+ cm, when presented with 1 gland equivalent of pygidial gland (either crushed or in hexane) or hexane control. Box and whisker plots illustrate: median (thick black), upper and lower quartiles (box), outliers (open circles).

source held about 0.5–1 cm above the surface. This behavior was released in both *Messor* species with pygidial gland secretions of the other species. Initially this was puzzling to us, because the chemical constituents of the glandular secretions in both species are quite different.

Neither benzaldehyde nor benzoic acid elicited any distinct behavioral response in *M. pergandei*. Workers of both species exhibited a slight attraction behavior to a point source of 3 μ l of 50, 10, and 5 ppm hexane solutions of pentadecane. However we did not document this behavior quantitatively because comparative tests with n-tridecane at the same concentrations showed that n-tridecane is considerably more effective. High n-tridecane concentrations (10+ ppm) elicited stronger aggressive behavior, rather than increased following. Therefore we used for the quantitative behavioral tests 3 μ l of a 5 ppm hexane solution (15 ng of n-tridecane). As shown in Fig. 6a significantly more *M. pergandei* workers near the nest exit were attracted to a n-tridecane applicator than to a control applicator in which the tip was contaminated with

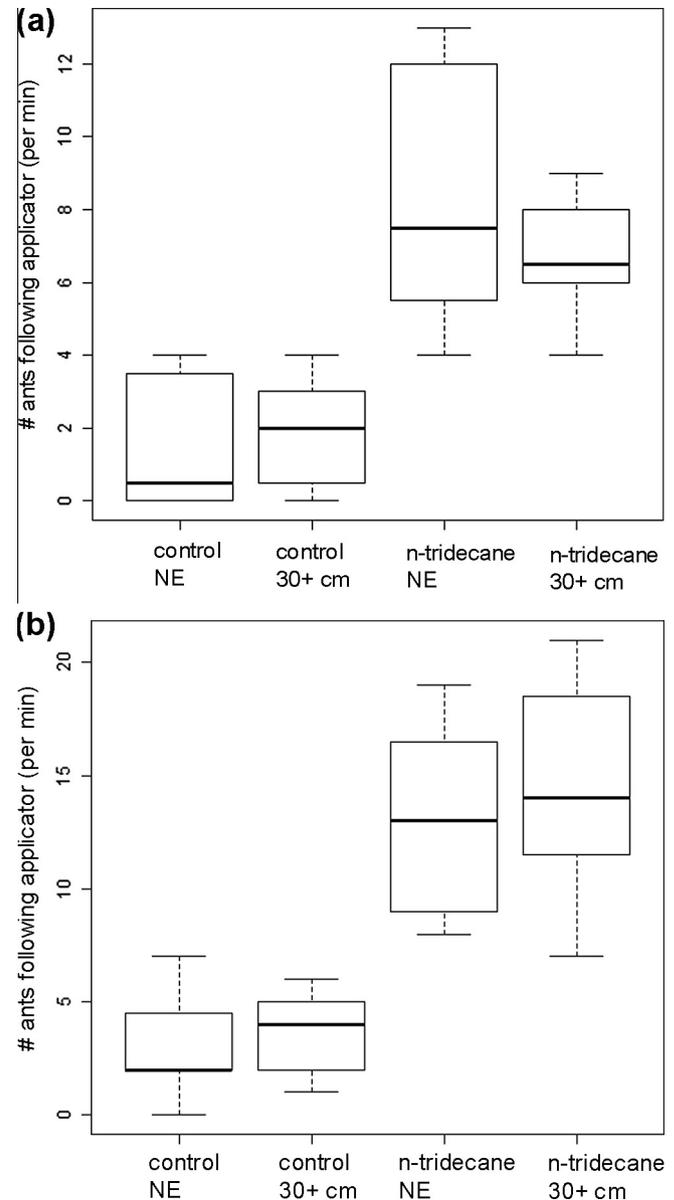


Fig. 6. Number of *M. pergandei* ($n = 8$) (a) or *M. andrei* ($n = 7$) (b) workers emerging from the nest entrance (NE) or following applicator stick for 30+ cm, when presented with either 15 ng of n-tridecane in 3 μ l of hexane, or a hexane control. Box and whisker plots illustrate: median (thick black), upper and lower quartiles (box).

3 μ l hexane (Wilcoxon rank sum, $W = 1$, $p < 0.01$, $n = 8$). When the applicator with n-tridecane was moved 30–40 cm from the nest entrance, there was no significant loss of ants, i.e. following was persistent (paired T -test, $t = 1.38$, $p = 0.21$, $n = 8$). Similar results were obtained with *M. andrei* (Fig. 6b: control v. n-tridecane at the nest entrance: T -test, $t = -5.13$, $p < 0.01$, $n = 7$; n-tridecane followers at nest entrance v. arena: paired t -test, $t = -1.08$, $p = 0.32$, $n = 7$). The response of both *M. andrei* and *M. pergandei* workers to n-tridecane was lower than the response to whole pygidial gland extracts. This suggests that the ants may respond to a blend of compounds.

4. Discussion

Messor is a moderate size genus of granivorous ants consisting of 113 described species worldwide (Bolton, 2012). The nine New

World species occur throughout western North America from southern Oregon to northern Mexico and west throughout the Great Basin and Sonoran Deserts (Johnson, 2001; Plowes et al., 2013). Three species of Nearctic *Messor* (*M. andrei*, *M. julianus*, *M. pergandei*) are group foragers that send out thousands of foragers that move in columns to harvesting sites with direction of the columns changing frequently, often every day (*Messor* foraging behavior reviewed in: Plowes et al., 2013).

Our study of the pygidial glands in New World *Messor* species revealed that only the three column foraging species possess a well-developed pygidial gland consisting of large paired clusters of glandular cells, large paired reservoir sacks, and a pair of distinct cuticle structures associated with the gland (Table 1). Histological investigations of *M. smithi*, and morphological inspection (including SEM studies) of the cuticle structures of the VIIth abdominal tergites and dissections of ethanol preserved specimens of *M. lariversi*, *M. stoddardi*, *M. chamberlini*, *M. chicoensis*, and *M. lobognathus*, indicated that these solitary foraging *Messor* species do not have or only have rudimentary pygidial glands.

The phylogenetic reconstruction by Bennett (2000) suggests that there are two clades of Nearctic *Messor*, each containing a mix of individual and column foraging species, and that the *Aphaenogaster albisetosa* group (formerly *Novomessor*) is the sister group to Nearctic *Messor*. The pygidial gland could be a synapomorphy for the clade including Nearctic *Messor* and the related *Aphaenogaster* species, alternatively the column foraging species may represent independent derivations of the gland. This raises the question as to why the three column foraging species are endowed with such a large pygidial gland. We hypothesize that the pygidial gland has an important role in the initiation of columns by group foraging Nearctic *Messor* species (Plowes et al., in preparation). Worker ants have the potential to control the release of pygidial gland contents by ventral flexion of the gaster. In the column foraging Palearctic *Messor* species *M. minor* and *M. wasmanni*, excitement and alarm behavior is elicited by putative tergal glands (Grasso et al., 1999). The structure, chemical constituents, and precise behavioral functions of the tergal glands in *M. minor* and *M. wasmanni* are still unknown.

In the current study we analyzed the chemical contents and possible behavioral function of pygidial gland secretions in *M. andrei* and *M. pergandei*. Benzaldehyde was a major compound in the pygidial gland secretions in *M. pergandei* (Blum et al., 1969). This substance was previously found in this species by Blum et al. (1969) who reported to having extracted benzaldehyde from the mandibular glands. In our study, *M. pergandei* workers were systematically dissected by removing the abdomen before dissecting the mandibular glands to prevent contamination of mandibular glands by abdominal gland secretions. We did not find any trace of benzaldehyde in mandibular gland secretions of *M. pergandei* workers, instead we determined the pygidial gland as source of this substance. Like Blum et al. (1969), we were unable to observe any behavioral reaction of *M. pergandei* workers exposed to benzaldehyde. It has been suggested that this substance serves as defensive secretion, and indeed some studies demonstrate that benzaldehyde and also benzoic acid (which we also detected in the pygidial gland secretions) can be an effective repellent against other ant species (Braekman et al., 1982; Eisner et al., 2005). To our knowledge, benzaldehyde has been found in only two other ant species. Bellas and Hölldobler (1985) identified it as one of the 10 volatile compounds in the hypertrophied mandibular glands of an Australian *Polyrhachis* species, and Wheeler and Blum (cited in Blum and Hermann, 1978) detected it in the pygidial glands of the dolichoderine *Azteca* sp.

n-Tridecane was the major compound found in the pygidial gland secretions in both *M. pergandei* and *M. andrei*. This compound is a common hydrocarbon previously detected in numerous ant

species belonging to different subfamilies, including species in the poneromorph subfamilies (e.g. Morgan et al., 2003), the Formicinae (e.g. Ali et al., 1988; Bellas and Hölldobler, 1985; Bergström and Löfqvist, 1968, 1973; Hefetz and Lloyd, 1982; Walter et al., 1993), and in a wide variety of myrmicine ants, including several species of *Messor* and *Pogonomyrmex* (e.g. Ali et al., 1989; Co et al., 2003; Hölldobler et al., 2004; Do Nascimento et al., 1993). In all these cases n-tridecane has been found in the Dufour glands. We have not investigated the contents of the Dufour gland in *M. pergandei* and *M. andrei*, however, we paid special attention during dissections to not contaminate pygidial gland secretions with Dufour gland contents.

Very little is known about the behavioral function of n-tridecane in ants. In *Formica lugubris* it appears to be an essential component of the blend of three compounds that comprise the sex pheromone (Walter et al., 1993). While n-tridecane has been identified in the Dufour gland of two Palearctic *Messor* species (*M. minor*, *M. capitatus*), and the Dufour gland has been demonstrated to elicit recruitment at nest entrances in both species, n-tridecane has not been confirmed as the active component of trail pheromone (Di Tullio et al., 2003). The current study shows that n-tridecane, secreted by the pygidial gland, functions as a powerful alarm-recruitment pheromone in *M. pergandei* and *M. andrei*. We hypothesize that n-tridecane might also be employed by column foraging New World *Messor* species during group initiation of column foraging activities inside the nest and at the nest entrance. Future work will focus on this question.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2013.06.006>.

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