# Odor-mediated group organisation and coordination in the termite raiding ant *Megaponera analis* (Mayr)

Abdullahi A. Yusuf [1, *+], Erik T. Frank[3+], Ayuka T. Fombong[2], Christian W. W. Pirk[1], Robin M. Crewe[1], Thomas Schmitt[4], Martin Strube-Bloss[5], Ian Gordon[6] and Baldwyn Torto[1,2]

[1] Social Insects Research Group, Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield, 0028, Pretoria, South Africa.
[2] International Centre of Insect Physiology and Ecology (*icipe*), P. O. Box 30772-00100, GPO, Nairobi, Kenya.
[3] Department of Ecology and Evolution, University of Lausanne, Lausanne, CH-1015, Switzerland
[4] Department of Animal Ecology and Tropical Biology, University of Würzburg, Würzburg, D-97074, Germany
[5] Department of Biological Cybernetics, University of Bielefeld, Bielefeld, D-33615, Germany
[6] BirdLife International Kigali Office, Box 2527, Kigali Post Office, Nyarugenge, Kigali, Rwanda

*Author for correspondence (e-mail: abdullahi.yusuf@up.ac.za)

+ Shared first co-authorship

**Abstract**

Visual and olfactory communication are vital for coordinated group hunting in most animals. To hunt for prey, the group raiding termite specialist ant *Megaponera analis*, which lacks good vision must first confirm the presence or absence of conspecific raiders. Here we show that, *M. analis* uses olfactory cues for intra-specific communication and showed greater preference for conspecific odors over clean air (blank) or odors from its termite prey. Chemical analysis of ant volatiles identified predominantly short-chained hydrocarbons. Electrophysiological analysis revealed differential sensory detection of the odor compounds, which were confirmed in behavioural olfactometric choice assays with odor bouquets collected from major and minor castes, and the two most dominant volatiles *n*-undecane and *n-tridecane*. A comparative analysis of the cuticular hydrocarbon profile with those of the short-chained odor bouquet of different populations shows a high divergence in the long chained profile and a much-conserved short-chained odor bouquet. This suggests that there is less selection pressure for divergence and individual recognition in the short- than the long-chained odor profiles. We conclude that olfactory communication serves as an alternative to visual or sound communication especially during group raids in *M. analis* when ants are not in direct contact with one another.

**Key words**- Cuticular hydrocarbons, foraging, group hunt, olfactory communication, *Pachycondyla analis*,

## 1. INTRODUCTION

Cooperative hunting allows predatory species to find and subdue larger or more numerous prey with greater efficiency than when foraging alone. It is believed to play a central role in the evolution of sociality and advanced cognitive abilities (Bailey et al. 2013) and necessitates a high degree of communication (Lang and Farine 2017). In most taxa this information transfer is conveyed visually or vocally to indicate position, presence/absence of group members and the onset of the hunt. These modes of communication during a hunt have been studied in a wide range of taxa, such as Lionfish (Lonnstedt et al. 2014), Carnivora (Bailey et al. 2013) or chimpanzees (Boesch 1994). In the absence of these senses, different modes of communication have evolved to improve group hunting, like eavesdropping in echolocating bats (Dechmann et al. 2009).

In ants, visual cues are rarely used for intra specific communication but mainly used for orientation and landmark navigation (Wehner et al. 2014). Vocal cues are also limited to stridulatory sounds mostly used as a distress signal. Where vision and sounds provide too limited information, chemical communication forms the major means of communication in eusocial insects and plays a central role in the organisation of their societies (Hölldobler and Wilson 1990). These roles include bringing individuals temporarily together at relevant locations and recruiting colony members for efficient utilisation of available resources. Collective exploitation, aggressive and/or defensive behaviours are also controlled by chemical signals with such behavioural traits presumed to be crucial in ecological dominance in invasive ant species (Holway et al. 2002). Recognising and discriminating between group and non-group members constitutes a very important ecological trait in ant societies as non-group members pose a serious threat to colony resources and ultimately its survival (Guerrieri et al. 2009, Yusuf et al 2010).

Social insects are extremely effective at coordinating their movements in time and space to optimise foraging patterns in stochastic environments. In most cases these food sources necessitate only a limited amount of coordinated cooperation for exploitation in order to forage optimally especially in solitary hunting species. However, this is not so for specialist group hunting species where there is the need to make quick, coordinated decisions. One such specialist is the termite raiding ant *Megaponera analis.*

*Megaponera analis* (Mayr) is a specialised termite predator, widely distributed in sub-Saharan Africa (Levieux 1966, Schmidt and Shattuck 2014, Frank and Linsenmair 2017). This ant species, commonly referred to as 'Matabele ants', organises highly coordinated group raids on termite species that belong to the subfamily Macrotermitinae (Frank and Linsenmair 2017a, Longhurst et al. 1978). After a scout has found a suitable food source, it will recruit 200-800 individuals to a site where termites are foraging. These ants will follow the scout in a distinct column formation with a van- and rear-guard (Frank and Linsenmair 2017b). Shortly before arrival, the raiders will wait for the column to gather and attack together. The hunting phase itself is divided into three; a first phase of breaking through the protective soil cover over the termite galleries. A second phase, in which the smaller ants rush into the openings to kill and carry out the prey and a third phase when the immobilised termites are gathered by the larger ants. The different phases rarely last longer than 4-5 min after which the ants gather again at the starting point of the raid, preparing to return together in the same column formation to the nest (Longhurst et al. 1978, Frank and Linsenmair 2017b, Yusuf et al. 2014a, Lepage et al. 1981, Bayliss and Fielding 2002).

Chemical communication within and outside the ant's nest, particularly during raids on termites has not been well described in *M. analis*. Previous studies reported the use of trail laying signals possibly released from glandular sources for communication

(Longhurst et al. 1978, Holldobler et al. 1994, Janssen et al. 1995). Recently, we showed that cuticular hydrocarbons mediated nestmate recognition in this ant species (Yusuf et al. 2010). In addition, injured ants attract helpers after the hunt to their location through pheromones in the mandibular gland (Frank et al. 2017). However, the well-coordinated raiding behaviour of *M. analis* on termite foraging sites suggests the involvement of a complex chemical communication system for both nestmate and caste recognition, and to some extent task allocations. This coupled with the observations that different worker castes played different roles during raids prompted us to explore the possible involvement of olfactory cues in raid coordination of *M. analis* workers. Here, we show that airborne volatile odors, mainly short chained hydrocarbons, continuously emitted by the ants, are involved in communication between raiding ants and is vital for the coordination of the raid.

## 2. MATERIALS AND METHODS

**Insects.** Three colonies of *M. analis,* identified by Marcus Stüben (University of Würzburg, Germany), with representatives of both sexes (workers and males), eggs, cocoons, and larva, were excavated at Mpala (0°17'N, 37°52'E), on the research facility of Mpala Wild Life Foundation Central Kenya. These were transported to the Animal Rearing and Containment Unit (ARCU) located on the *icipe* Duduville campus in Nairobi (-1°22′S, 36°89′E), Kenya. The ant colonies were placed in nesting boxes (20 cm × 20 cm × 20 cm) made of aluminium with a removable lid to observe the colony (Yusuf et al. 2013). The base of the nesting box was partially filled with soil from the excavated ant's nest that served as nesting material. This was attached to a foraging arena (1.0 m × 1.5 m) made of Perspex that was also partially filled with sterilised soil. This soil was thoroughly washed with double-distilled water and oven-dried overnight at 160 °C.

Ants were fed with termites (mainly of the sub-family Macrotermitinae) collected from mounds or foraging galleries around the *icipe* Duduville campus. Feeding was carried out twice daily in the morning and evening. Conditions in the rearing room were kept between 50 - 60% relative humidity and 24 – 29$^{o}$C under a natural photoperiodic cycle of 12L: 12 D.

For comparative chemical analyses, ants were also collected from a population in the Comoé National park, Côte d'Ivoire, in the vicinity of the Comoé National Park Research Station (8°46'N, 3°47'W) and from a population in Gorongosa National park (18°58'S, 34°21'E), Mozambique in the vicinity of the E.O. Wilson laboratory.

**Classification of workers.** Since workers of *M. analis* exhibit monophasic allometry (Villet 1990, Crewe et al. 1984), workers were classified into two groups (major and minor) using the following morphological characters: interocular distance, body, and scape length as described (Crewe et al. 1984). Workers falling into classes 1 to 5 were grouped as minor and those in classes 6 to 10 as major workers.

**Volatile collections**

To determine the olfactory cues used for inter-caste communication by *M. analis* workers, volatiles were collected separately from major and minor workers and a combination of the two castes in the following treatments: a) 20 majors; b) 10 majors: 10 minors (mixed workers); c) 20 minors. The ants were placed in 2L cylindrical glass containers with single-port lids (Analytical Research Systems INC, Gainesville, FL, USA) and charcoal-purified humidified air was passed over the ants and through a Super Q trap (30 mg) at 0.5 L/min for 22 hr as described in (Yusuf et al. 2014b). Prior to connecting the adsorbent traps, the collection chambers were purged by passing humidified air through them for 20 min to allow the ants to settle down in the containers, and to flush out potential alarm

pheromones released during handling of the ants. Each filter was eluted with 200 µL of GC-grade dichloromethane (Sigma Aldrich St. Louis, MO, USA) and the eluent stored at –20 °C prior to analysis. This procedure was repeated using worker ants from three different colonies.

The cuticular hydrocarbon profile of workers from Côte d'Ivoire and Mozambique were extracted for 10 min in 1 ml of Hexane. Afterwards the extracts were transported to the University of Würzburg (Germany) and stored at -20 °C until use.

**Analyses of odors.** To determine the qualitative and quantitative composition of odors and cuticular hydrocarbons from different caste and populations, volatile and cuticular hydrocarbon extracts were analysed by coupled gas chromatography-mass spectrometry as follows.

Ant volatiles**:** Coupled gas chromatography-mass spectrometric analysis of the Super Q volatile extracts of workers were carried out on an Agilent Technologies 7890A GC equipped with an HP-5 MS capillary column (30 × 0.25 mm ID × 0.25 µm film thickness) coupled to a 5795C MS. One microliter of each sample was injected into the GC in a splitless mode, with helium used as the carrier gas at a flowrate of 1.0 mL/min. The oven temperature was 35°C for 5 min, increased to 280 °C at 10 °C/min, and then held at this temperature for 15 min. Spectra were recorded at 70 eV in the electron impact (EI) ionisation mode. Compounds in the ant volatiles were identified by comparing their mass spectral data with those in the library (NIST11). The identities of the *n*-alkanes were confirmed by co-injection and comparison of MS data with those of authentic standards. Compounds present in the volatiles were quantified using 1-heptadecene as an internal standard.

Cuticular hydrocarbons: Cuticular hydrocarbon (CHC) extracts were evaporated to a volume of approximately 100 µL and 1 µL was analysed by using a 6890 gas chromatograph coupled to a 5975 mass selective detector Agilent Technologies (Waldbronn, Germany). The GC was equipped with a DB-5 capillary column (0.25 mm ID × 30 m; film thickness 0.25 µm, J & W Scientific, Folsom, Ca, USA). Helium was used as a carrier gas at a flow rate of 1 ml/min. A temperature program from 60°C to 300°C with 5°C/min and finally 10 min at 300°C was employed. The mass spectra were recorded in the EI mode with an ionization voltage of 70 eV and a source temperature of 230°C.

The software ChemStation version (Agilent Technologies, Waldbronn, Germany) for windows was used for data acquisition. Identification of the components was accomplished by comparison of library data (NIST 11) with mass spectral data of commercially purchased standards and diagnostic ions.

**Chemicals.** *n*-nonane, *n*-decane, *n*-undecane and *n*-dodecane with purity of >99% were obtained from Sigma Aldrich Chemical Company (Gillingham, Dorset, UK). *n*-tridecane, *n*-tetradecane, *n*-pentadecane and *n*-hexadecane were provided by the late Dr. Peter Teal, USDA/ARS-Centre for Medical, Agricultural and Veterinary Entomology, Gainesville, Florida, USA.

**Olfactometer.** The olfactometer consisted of a glass Y-tube (base, 7.5 cm long; Y-arms, each 7.5 cm long; internal tube, 10 mm outer diameter). The Y-tube apparatus was modified after the design of (Caroll et al. 2006) and bioassays were conducted as previously reported in Yusuf et al. (2014b) with slight modifications. Briefly, the two arms and base tube of the olfactometer were connected to Teflon tubes similar in size and were in turn attached directly to the odor source and vacuum pump. A mesh screen was placed at each end of the olfactometer to prevent ants from entering the Teflon tubes.

Odor sources were placed in 200 ml glass chambers with screw tops containing inlets for incoming air and outlets for odors to exit into the Y-tube. Charcoal-purified air was passed into the odor chambers at a flow rate of 250 ml/min. One of the Y-arms was connected to an odor source while the other was connected to an empty jar with only clean air (blank) passing through it. The odors were extracted through the base arm at 500 ml/min by a vacuum pump to ensure a constant flow and to prevent odors from building up in the Y-tube. A score line was drawn on the two arms of the olfactometer at 2 cm from the Y-tube junction.

**Bioassays with living ants.** To answer the question if olfactory cues are used by ants for communication, we tested the responses of major and minor workers of *M. analis* to conspecific odors in a Y-tube olfactometer. The odor sources consisted of 20 (a) major (b) minor and (c) a combination of 10 major and 10 minor workers against clean air used as control. To rule out possible bias in relation to the limited choices of odors presented, we tested the response of workers to their own odors against those of termites in their galleries (positive control), an odor source to which the ants respond (Yusuf et al 2014b). The bioassays were conducted at room temperature (24 ± 1°C) and 50 - 60 % RH. In order to conform to the circadian rhythms of foraging and raiding behaviour, all bioassays were carried out in the mornings and evenings (during the period 0700 - 1000 hr and 1600 - 1730 hr local time, respectively) using ants from three different colonies. Prior to bioassays, the odor source chambers with ants were purged by passing air through them for 10 min to allow the ants to settle down in the containers, and to flush out potential alarm pheromones released during handling of the ants.

Test ants were introduced individually by disconnecting the Y-tube at its base and allowing the ant to walk into the olfactometer. Subsequently, the tube was reconnected to re-establish airflow from the odor sources through the arms and out at the base towards

the vacuum pump. The ant was allowed to settle down for 5 min, after which its behaviour was monitored. A choice was recorded when an ant stayed for at least 1 min in an arm or when it frequently visited an arm. A no-choice response was recorded when the ant remained in the base arm for more than 5 min. Each test was terminated 10 min after the introduction of the ant into the Y-tube. Sixty replicates were carried out for each treatment (30 minor and 30 major workers). To avoid positional bias, odor chambers were switched for every replicate. A clean Y-tube was used for each replicate in order to avoid contamination from trail pheromones. All the Y-tubes were thoroughly cleaned with a scent free detergent and water, rinsed with acetone, and dried in the oven for five hours at 160°C. The Teflon tubes were washed with water and detergent, rinsed with double-distilled water, and then dried in a stream of nitrogen.

**Bioassays with volatile extracts.** Next, we tested whether ant responses to volatiles was dose-dependent. The number of ants used for this collection was based on the results obtained from the analysis of volatiles collected from living ants. Volatiles were collected from 50 worker ants, comprised of 25 majors and 25 minors for 8 hr using procedures described earlier with living ants. The adsorbent trap was eluted with 400 µL of dichloromethane. Five concentrations including 12.5, 25, 50 and 100 ant hours (an ant hour is equivalent to the amount of volatiles released by a major or minor ant in an hour) were prepared from the stock solution. Each dose was, loaded on to a rubber septum, air-dried and then transferred into a 200 mL glass container and tested individually as an odor source against clean air (control). Forty ants (20 majors, 20 minors) were used for each concentration making a total 160 ants from three colonies for the assays.

**Bioassays with *n*-undecane and *n*-tridecane.** Previously *n*-undecane and *n*-tridecane were shown to elicit alarm responses in workers of *M. analis* in the field (Longhurst et al. 1979). To demonstrate the roles of these compounds in olfactory communication in

worker castes, we tested responses of major and minor ants to these two major components identified in their volatiles at varying concentrations; 0.125, 0.25, 0.5 and 1.0 ng/septa in the olfactometer as previously described. Preparation of the different of each compound was done similarly as described for the extracts. For each concentration of synthetic compound tested, responses of 40 ants (20 majors and 20 minors) were recorded and 320 ants from three different colonies used for these assays.

**Electroantennography (EAG).** To determine if volatiles from workers are detected by ant antennae, electroantennographic analyses were performed with the extracts and the elicited responses recorded. A silver wire inserted into the ant's head capsule served as reference electrode. A glass capillary filled with potassium-chloride solution and connected to a silver wire was positioned at the tip of the antennae, which was previously cut off. The electrode signal was 10× pre-amplified at the head stage (Neuroprobe Amplifier 1600, A-M Systems, Sequim, USA), high-pass filtered (Kemo VBF 8, Kemo Inc., Greenville, USA) and digitalised by an acquisition board (Labtrax 4/16, WPI, Sarasota, USA). Data were recorded with LabScribe 3 (WPI, Sarasota, USA) at a sampling rate of 1 kHz.

The compounds were applied using a stimulus controller (Stimulus Controller CS-55, Syntech, Hilversum, The Netherlands) generating a continuous airflow of 1 L/min added with a stimulus flow of 0.5 L/min. Two stimulus chambers were inserted into the air stream (stimulus chamber one and two). Prior to odor stimulation, an airflow of 0.5 L/min was blown over an untreated filter paper placed in stimulus chamber one. For providing the stimulus, the airflow switched from stimulus chamber one to stimulus chamber two, equipped with a filter paper treated with the test compound. After 0.5 seconds of stimulation, the airflow was switched back to stimulus chamber one. All stimuli were presented three times per individual in a pseudorandomised order.

**Statistical analyses.** To visualise the data for chemicals from the three populations of *M. analis* a Non-metric multidimensional scaling (NMDS) was used. A permutational Multivariate Analysis of Variance using Distance Matrices using the ADONIS function in R was performed to test for differences between populations and colonies of *M. analis*. To test if ants exhibited sensory responses to C10-C17 hydrocarbons, data for the EAG was subjected to a Wilcoxon Rank Sum test and the contributions of these compounds to the EAG visualise using a Principal Component Analysis (PCA). Data for the different bioassays were analysed using a one-sample $\chi^2$ test where the number of ants responding to the test odor source was compared to those responding to the control (clean air) or termite gallery odor where appropriate. Non-responding were excluded from all analysis to preclude bias as they do not contribute to the test. All statistical analyses were carried out using R 2.12.0 (R Development Core Team, 2010).

## 3. RESULTS

**Analysis of volatiles.** Volatiles were released by all worker castes of *M. analis*. Analysis of these volatiles revealed that major, minor and mixed (minors: majors) worker groups produced volatiles that were identical in composition. These comprised mainly of straight- and branched- chain saturated hydrocarbons, with chain lengths between $C_9$ (nonane) and $C_{17}$ (heptadecane) (Fig 1; Fig S1). However, the proportions of these compounds varied between castes and population (Fig 1). The volatile production pattern was similar for the colonies from Kenya and the two populations of *M. analis* from Mozambique and Côte d'Ivoire. The profiles showed similar qualitative composition of straight- and branched- saturated hydrocarbons, with *n*-undecane (C11) and *n*-tridecane (C13) being the major components (Fig 1 and Table S1).

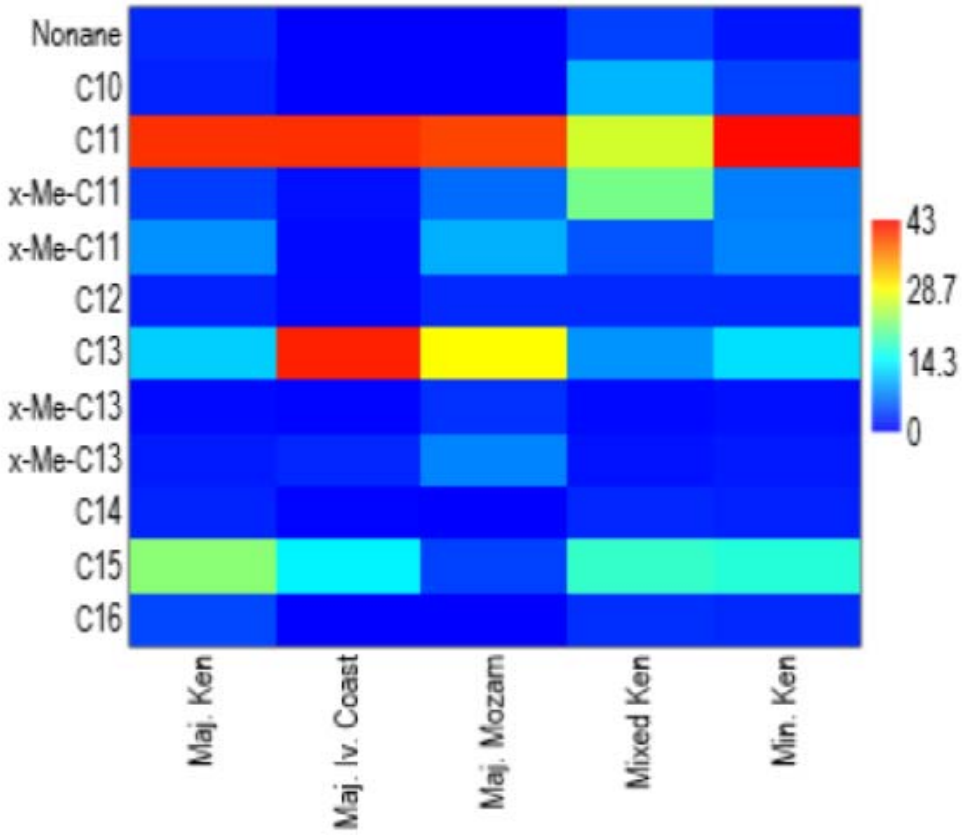


**Fig 1** Composition of individual components from volatiles and cuticular hydrocarbons from different populations of *Megaponera analis.* Maj. Ken (Major workers from Kenya), Maj. Iv. Coast (Major worker from Cote d' Ivoire), Maj. Mozan (Major worker from Mozambique), Mixed Ken (Mixed workers from Kenya) and Min. Ken (Minor workers from Kenyan). Individual components are shown on y-axis; C10-C17 are Decane, Undecane, Dodecane, Tridecane, Tetradecane, Pentadecane, Hexadecane and Heptadecane. While x-Me-C11 and x-Me-C13 are methyl branched Undecane and methyl Tridecane respectively

**Differences in cuticular and odor profiles between colonies and populations.** To determine whether the cuticular hydrocarbons of the different ant populations were conserved, volatile and cuticular hydrocarbon (CHC) profiles of *M. analis* from Kenya, Côte d'Ivoire and Mozambique were analysed. The chemical profiles identified individual groups and their colonies of origin for the ant populations from Côte d'Ivoire

and Mozambican (Fig 2; ADONIS: Côte d'Ivoire: $F_{4,20}$=33, $R^2$=0.89, p<0.001, Mozambique: $F_{4,24}$=5.68, $R^2$=0.53, p<0.001). The CHC profiles showed qualitative and quantitative differences (Fig 2 ADONIS: Population: $F_{2,50}$=134.8, $R^2$=0.85, p<0.001). These differences were less apparent in the odor plume of *M. analis* from Kenya, explaining only 25% of the variance compared to 85% in the CHC profile (Fig 2 ADONIS: Population: $F_{1,34}$=11.3, $R^2$=0.25, p<0.001). Whereas minor differences were detected between the odor plumes of colonies in the Côte d'Ivoire populations (Fig 2, ADONIS: Colony: $F_{2,10}$=3.96 $R^2$=0.49, p>0.05), no significant differences were detected in the odor plumes of the Mozambican populations (Fig 2, ADONIS: Colony: $F_{4,23}$=0.94, $R^2$=0.16, p>0.05).

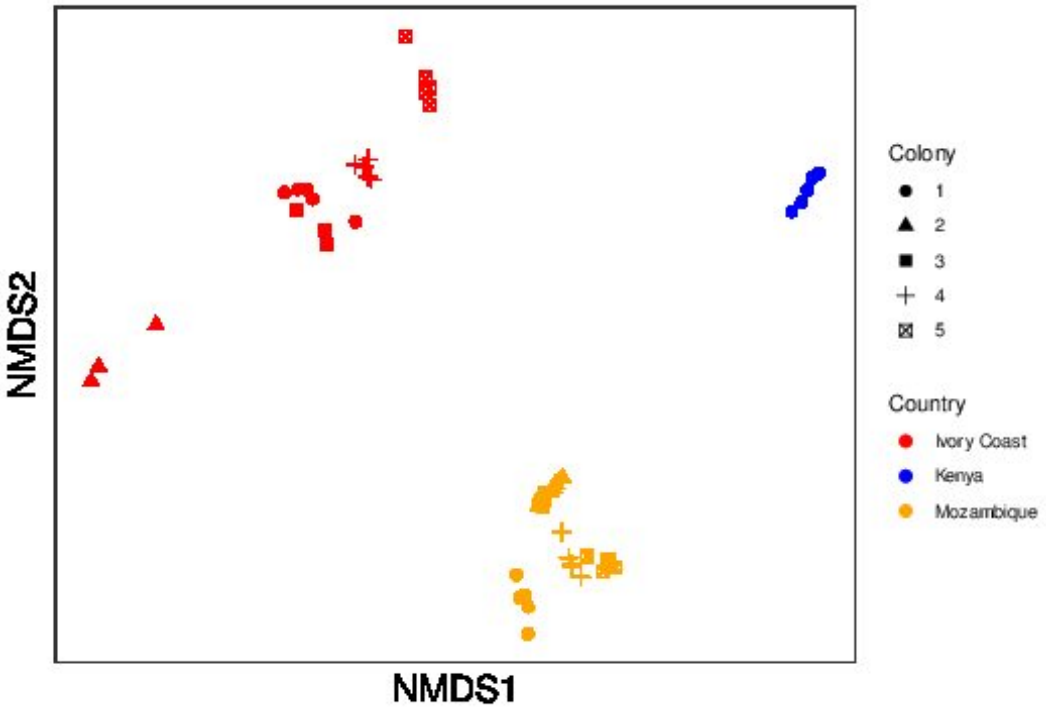


**Fig 2** A non-metric multidimensional scaling (NMDS) plot of the chemical profiles comparing populations and colonies from Côte d'Ivoire (n=21), Mozambique (n=25) and Kenya (n=5).

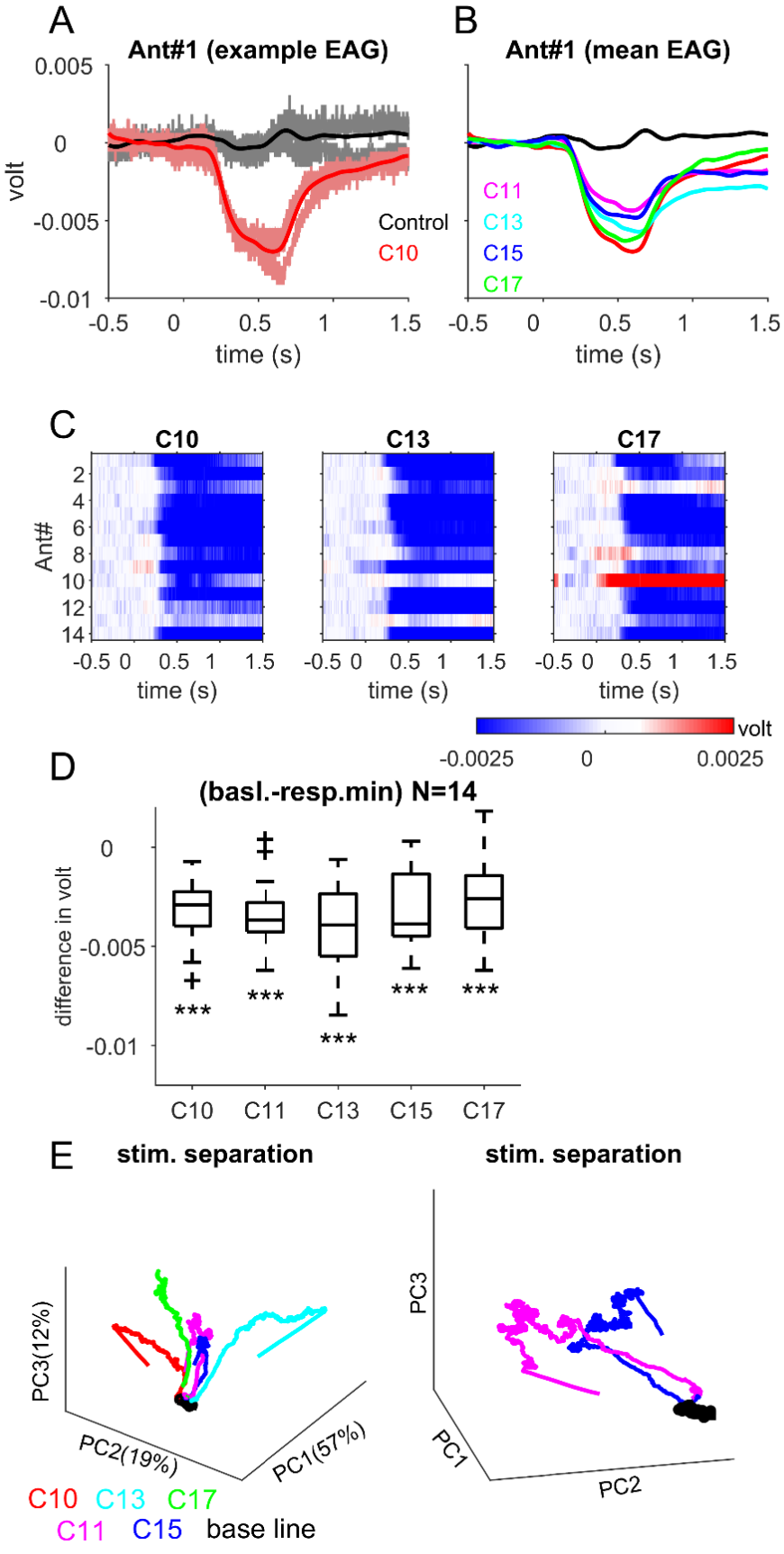
A
Ant#1 (example EAG)
0.005
0
-0.005
-0.01
volt
Control
C10
-0.5 0 0.5 1 1.5
time (s)
B
Ant#1 (mean EAG)
C11
C13
C15
C17
-0.5 0 0.5 1 1.5
time (s)
C
C10
C13
C17
Ant#
2
4
6
8
10
12
14
-0.5 0 0.5 1 1.5
time (s)
volt
-0.0025
0
0.0025
D
(basl.-resp.min) N=14
difference in volt
0
-0.005
-0.01
***
C10 C11 C13 C15 C17
E
stim. separation
PC3(12%)
PC2(19%)
PC1(57%)
C10 C13 C17
C11 C15 base line
stim. separation
PC3
PC1
PC2

**Fig 3.** EAG recordings of ants' responses to C10-C17 odors**.** All tested straight-chained hydrocarbons elicited significant antennal responses. **A|** The EAG signals of three C10 repetitions (pink) and their average (red) is shown. The three replicates of the control (grey) and their average (black) remained at the baseline. Stimulation started at time zero and last for 500 ms. **B|** The three repetitions of each odor were averaged in each insect (the shown example is the same as in A). **C|** Overview of the 14 insects tested. Each line corresponds to the colour coded mean activity of the three replicates per stimulus. Stimulation induced typically negative EAG signals. **D|** In each insect we calculated the difference between the minimal baseline activity in the 500 ms before odor onset and the stimulus induced EAG minimal d in the first 1000 ms of stimulation. The distribution for all stimuli was significantly different from zero (singed rank test; $p<0.001$). **E|** The population vectors (as shown in C) of all stimuli were used in a principal component analysis. Plotting the first three principal components revealed distinct EAG activity for all tested molecules. The variation explained by the first principal components is given in the axes. An enlargement of and slight turn of the 3D plot is shown to illustrate the separation between C11 and C15 (right).

**Sensory response to short chained hydrocarbons.** Electrophysiological assays showed that *n*-decane (C10), *n*-undecane (C11), *n*-tridecane (C13), *n*-pentadecane (C15) and *n*-heptadecane (C17)) elicited significant (signed rank test; $p<0.001$) antennal responses (Fig 3A, B, C, D). A plot of the first three principal components of the recorded EAG responses indicated that the different hydrocarbons induced distinct receptor activity and together the C10-C17 accounted for 88% of these differences (Fig 2E).

**Behavioural response to ant volatiles**. To answer the question if ants use olfactory cues from conspecifics and different castes for intra- and inter-specific communication during raids or in their nest, we tested the responses of ants to odors from different castes. Both major and minor workers responded to odors of their conspecifics (Fig. 4). Overall, more than 70% of the ants chose the odors from the mixed conspecifics (Fig 4A). The responses of major and minor workers to odors of the mixed workers were significantly higher than the responses to control (clean air) ($\chi^2 = = 9.8$, DF = 1, $N = 30$, $p < 0.01$, majors; and $\chi^2 = 14.4$, DF = 1, $N = 30$, $p < 0.001$, minors). Similarly, the responses of major workers to their own odors ($\chi^2 = 6.8$, $N = 30$, $p < 0.01$), and minor workers to their own volatiles ($\chi^2 = 14.29$, DF = 1, $N = 30$, $p < 0.001$) were significantly higher than responses of the ant castes to the control (clean air) (Figs 4B and 4C). When workers were presented with a

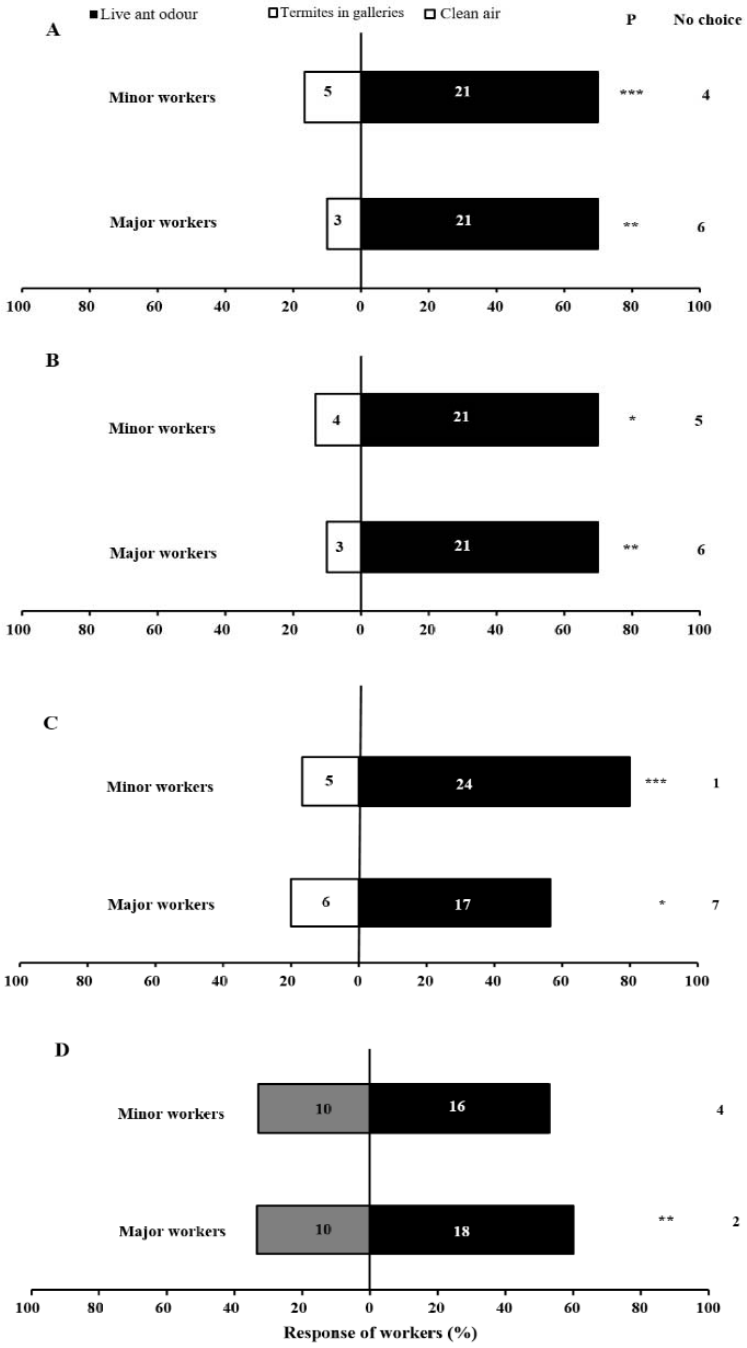


**Fig 4** Preferences of minor and major workers of *M. analis* for odors of (A) mixed groups of ants (major and minor), (B) Major workers, (C) Minor workers against the control clean air (open bars) and (D) between odors from mixed workers and termites in galleries. Numbers within bars represent the number of ants making a choice, while numbers outside bars refer to ants that made no choice in the assay, $N$= 180, 90 each for major and minor workers in each treatment, * $p <$ 0.05, ** $p < 0.01$, ***$p < 0.001$).

choice between their own odors and those from their termite prey (Fig. 4D), they showed a preference for conspecific odors, especially for major workers ($\chi^2 = 3.80$, DF = 1, $N$ = 60, $p < 0.01$). Because responses of both major and minor workers to odors from conspecifics were similar to those to the control (clean air), the data for all workers from subsequent assays were pooled together.

**Behavioural responses of workers to different doses of volatile odors.** To determine whether responses of workers to volatiles was adaptive, we tested the responses of workers to different concentrations of ant volatiles in Y-tube assays. All workers responded to the concentrations of ant volatiles preferring these over the control (clean air) (Fig 5) $\chi^2$ (DF = 1, $N$= 160, $p < 0.001$).

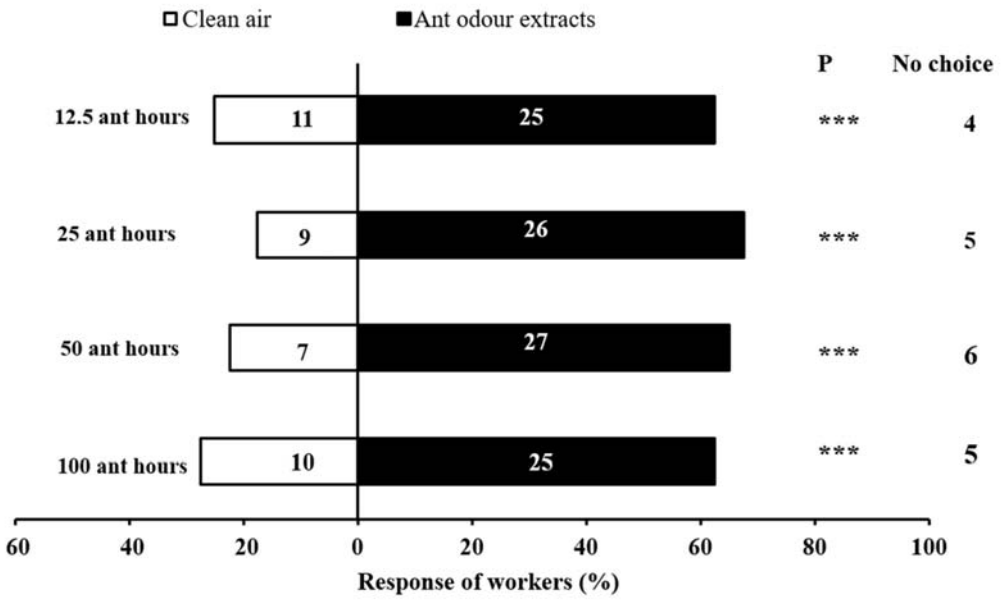


**Fig 5** Preferences of workers of *M. analis* for different concentrations of conspecific odors (12.5, 25, 50 and100 ant hours) against the control odor clean air (open bars). Numbers within bars represent the number of ants making a choice, while numbers outside bars refer to ants that made no choice in the assays. *N= 160,* 40 replicates for each treatment, *** = $p < 0.001$).

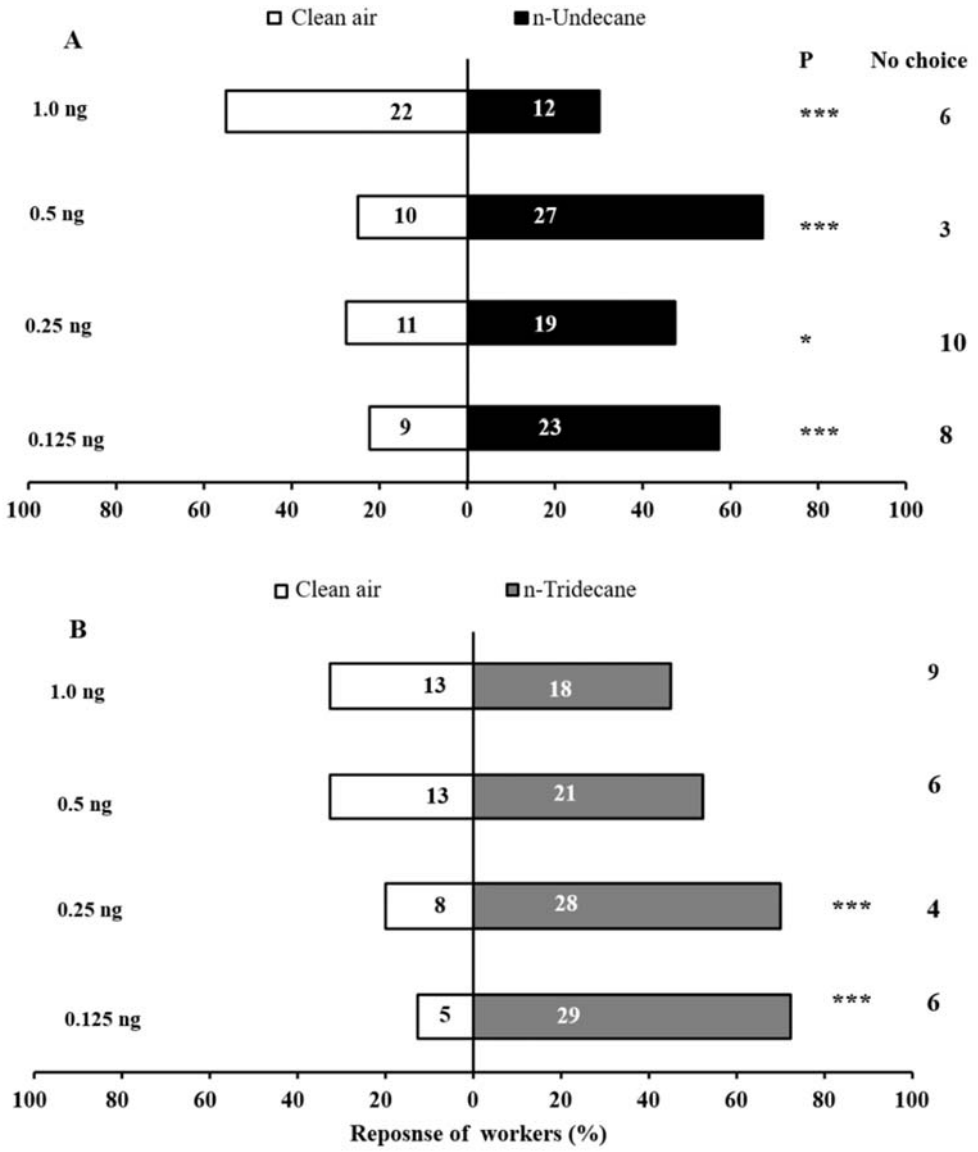


**Fig 6** Responses of *M. analis* workers to (A) *n*-Undecane and (B) *n*- Tridecane at 0.125, 0.25. 0.5, and 0.1 ng compared to the control clean air (open bars). Numbers within bars represents to the number of ants making a choice, while numbers outside bars refer to ants that made no choice, *N*= 320, 40 ants for each assay. Asterisks represent statistical significance (*$p < 0.05$, *** $p < 0.001$).

**Behavioural response to *n*-undecane and *n*-tridecane.** To demonstrate the roles of chemical components from the odor profiles of *M. analis,* we selected the two main components *n*-undecane and *n*-tridecane that were shown to be part of the ants alarm pheromones for dose-response tests against workers. Workers responded differently to *n*-

undecane and *n*-tridecane (Fig 6), preferring *n*-undecane to the control at all concentrations except at 1.0 ng (Fig 6A, $\chi^2 = 5.05$, DF = 1, $N = 30$, $p < 0.05$). However, they only preferred *n*-tridecane at 0.125 ng ($\chi^2 = 29.00$, DF = 1, $N = 30$, $p > 0.001$) and 0.25 ng ($\chi^2 = 19.95$, DF = 1, $N = 30$, $p > 0.001$) (Fig 6B).

## 4. DISCUSSION

The position of group members during a hunt is vital and generally mediated by acoustic or visual cues (Lang and Farine 2017). Here we show that, the group-hunting termite specialist *M. analis* uses short-chain hydrocarbons to communicate with nestmates over a short distance. This allows for a highly coordinated hunt that, to our knowledge, is unique among predatory ants like those in the genus *Leptogenys* spp and *Dorylus* spp.

**Odor plume composition and detection.** The results of the olfactometer assays show that volatiles mediate non-tactile conspecific communication in workers of *M. analis* suggesting that ants use volatiles to communicate their presence under group related foraging conditions. In our study, no significant changes were observed in the responses of major and minor workers to the volatiles of living major and minor ants, suggesting that ant odors may play a generalist role to signal presence or absence of nestmates. Ants also preferred conspecific odors over odors from cues they commonly encounter during raids such as termites in their galleries (Yusuf et al 2014b), indicating a greater sensitivity to detecting their own odors, especially as found in the current study for major workers. However, it is important to note that several factors may determine the quality and quantity of odors released from termites in a gallery including the presence or absence of food, temperature, humidity and composition of microbial community in the gallery. Thus, although our results show differential sensitivity between major and minor workers to the odors released from the termites in their gallery, and conspecific odors, further behavioural experiments are required to understand these interactions. Communication

and maintaining social cohesion among workers in *M. analis* is vital due to their dietary specialisation (termites), that is, highly coordinated group raiding behaviour and raid phases that change within minutes (Frank and Linsenmair 2017a, Yusuf et al. 2014a). Hence, they rely on a sophisticated communication system using pheromones to achieve the maximum reward during foraging. Earlier studies have reported similar chemical communication in some ants. For example, the attraction of adults of the red imported fire ant *Solenopsis invicta* to volatiles from conspecific larvae (Glancey and Dickens 1988) and an odor-based queen-queen recognition system in the hairy panther ant *Neoponera villosa* (D′Ettore and Heinze 2005).

Analysis of volatiles showed that odors emitted by major and minor workers were identical, comprising primarily saturated hydrocarbons whose quantities were greater in majors than minors. Previously, we had used cuticular hydrocarbon (CHCs) profiles to group different ant colonies successfully (Yusuf et al. 2010). Interestingly, the ant odor plume does not seem to allow accurate nestmate recognition and is highly conserved across populations in Africa (Mozambique, Côte d'Ivoire and Kenya), while the CHC profile varies greatly between these populations. We therefore believe that there was little evolutionary pressure for the odor plume to be used for nestmate recognition, but rather it is used as a conspecific recognition signal. During raids, contacts through direct interactions with non-nestmates are very rarely observed (pers. obs.) making it unnecessary to add complexity to the plume to achieve nestmate recognition. If the raiding group does indeed meet another colony, the normal CHC recognition profile should suffice for the colonies to identify non-nestmates.

The compound released in the highest proportion from the volatiles and CHC profiles of *M. analis* is *n*-Undecane. Interestingly, *n*-Undecane had previously been identified in the Dufour's gland secretions of *M. analis,* and reported as a potential alarm pheromone

(Longhurst et al. 1979). *n*-Undecane is also an alarm pheromone in some ant species like *Camponotus pennsylvanicus*, *C. herculeanus* (Ayre and Blum 1971), *C. obscuripes* (Fujiwara-Tsujii et al. 2006) and many Formicidae spp. (Verheggen et al. 2010). *n*-Pentadecane, another major component identified in the volatiles, is one of the major components in the Dufour's glands of the giant bull ant *Myrmecia gulosa* (Cavill and Williams 1967), Cape harvester ant *Messor capensis* (Brand and Mpuru 1993). These cross-generic similarities in the major alarm pheromone systems of ants suggest that they may have been biosynthesised early in the evolution of ant societies and have remained conserved over time.

However, to eliminate the possibility that the ants in our bioassays responded to an alarm rather than a task or communication pheromone, we tested different doses of *n*-Undecane and those of *n*-Tridecane, another alarm pheromone, respectively. In both tests, workers responded differently to different concentrations of the two compounds with preferences for these individual volatiles at low concentrations, but an alarm response was elicited in the ants at higher concentrations (Fig 6). Thus, odor-mediated behaviour in this ant species appears to be concentration-dependent and clearly suggests that odors may serve as task allocation cues. With odors released at concentrations above colony thresholds being perceived differently by different castes. Despite these findings, further research should look to test the other components identified in the volatiles, individually and as a blend to elucidate the role of the full spectrum of the volatiles released by workers of *M. analis.*

**Odor plume benefits during raids.** A constantly emitted odor plume by ants in raiding parties could be beneficial during raids. Previous studies have shown a clear formation within the raiding column, with ants even retaking a position at the front or tail of the column if displaced (Frank and Linsemair 2017b). Potential position dependent odors

within the column could be beneficial in identifying and maintaining column formation. The raid leader also halts the raiding party shortly before arriving at the hunting ground to gather the members of the column to raid as a coherent group. An increasing concentration of ant odor could be used as a cue to determine whether enough ants have gathered to start the next phase of the raid (Frank and Linsenmair 2017a and b).

During the raid itself, the raiders recognise the onset of the return journey with the odor decreasing in concentration on the hunting site and the ants gathering and waiting at the starting point of the raid. Ultimately, odors could explain the stark contrast in the behaviour of injured ants in the presence or absence of nestmates, even without direct contact. If nestmates are nearby, the injured ant stays mostly motionless or on its back, if nestmates are absent the injured ant will immediately start the return journey to the nest (Frank et al. 2018).

**Chemical communication in group foraging ants.** While group foraging is a common occurrence in social insects, it is rarely followed by such a complex coordinated hunt. Army ants for instance hunt in large groups comprising 100,000 individuals starting with one main trunk from the nest and branching out to cover the entire ground subduing prey as they encounter it and recruiting nestmates with secretions from their mandibular gland (Bruckner et al. 2018). This form of group foraging may not necessitate the use of an odor plume since it is relatively uncoordinated and differs from those of *M. analis*. First, army ants are in constant direct contact with one another during a raid, while *M. analis* workers at the hunting ground fan out into dozens of smaller "termite hotspot" hunting sites in an area of approximately $1m^2$ (Frank and Linsenmair 2017b). Secondly, foraging in army ants is devoid of clearly distinguishable hunting phases like coordinated recruitment, outward journey, duration at food source and return journeys. Army ants generally leave the nest and subdue prey as they encounter them, this opportunistic hunting approach

inhibits the development of a truly specialised hunting strategy with different phases as is the case in *M. analis* and necessitates less specific coordination between nestmates to succeed. Lastly, the hunt is not as temporally constrained as it is in *M. analis*. In army ants, a foraging bout can last up to 12 hours, while in *M. analis* it is often over after just 10 minutes (excluding travel time). A quick non-tactile information transfer for the position of nestmates in such a short time window could be essential. Furthermore, considering the high cost of producing and maintaining a volatile odor plume, it might be too costly for longer periods of time but essential for precision and success during shorter raids.

Apart from the Dorylinae, the other predatory subfamily in ants is the Ponerinae, to which *M. analis* belongs. The vast majority of ponerines are solitary hunters in which there would be no necessity for such an odor plume. *Megaponera analis* clearly stands out with their large group foraging strategy, continuous allometric size polymorphism, an unusually large colony size and an ergatoid queen, all of which are army ant like but unique adaptations for a ponerine species. While there are other ponerine species that hunt in groups, notably in the genus *Leptogenys* and *Neoponera*, in many cases this is to subdue larger prey (like millipedes or Isopoda), where the benefit would be minimal i.e. restricted to local recruitment. There are some species of *Leptogenys* and *Neoponera* which show a similar hunting strategy as *M. analis* towards termites, albeit on a smaller scale. We expect an odor plume to have a similar benefit in these species as well. Further studies into the hunting behaviour and chemical communication of these species could provide interesting leads on its evolution.

## 5. CONCLUSION

In this study, we identified 12 hydrocarbons and tested responses of ants to two previously identified components reported as alarm pheromones emitted by the ants. Moreover, the

recorded EAG responses suggest that the different hydrocarbons may induce distinct receptor activity. We found behavioural response in *M. analis* to be both concentration- and caste-dependent.

Finally, we demonstrated odor-based communication in *M. analis*, and its possible role in task allocation thereby contributing to social cohesion. We propose that this odor-based short distance communication system might have evolved as an alternative to visual or acoustic communication in a group hunting species with a high demand for quick differentiated and coordinated behaviours during their foraging phase.

**Conflict of Interests**

The authors declare no conflict of interests.

**Funding**

Funding for this research was provided in parts by the Dutch SII project 2004/09 Activity No. 10799 to *icipe*. DAAD in-region scholarship to AAY, University of Pretoria, South African National Research Foundation's CPRR to CWWP and RMC. The Alexander von Humboldt Foundation's Georg Foster HERMES Research Fellowship for Experienced Researchers (grant number 3.4-NGA-1164298), Tandem Research Fellowship (grant number ZAF-1164298 –GFHERMES-E) and the South African NRF's Development funding for Y-rated researchers (grant number 116347) to AAY.

***Acknowledgments***- We thank Mark J. Carroll of the Chemistry Research Unit, USDA/ARS-CMAVE, Florida, USA for providing the Y-tubes, Raphael Erangae of Mpala Research Centre, Kenya for his help in excavating ant colonies and Peter Malusi of *icipe* for assisting in maintaining ant colonies in the laboratory, Mpala Research Centre Nanyuki, Kenya for permission to excavate ant colonies. We thank Florens Fischer for his help collecting the EAG data.

## REFERENCES


Ayre, G., Blum, M. 1971. Attraction and Alarm of Ants (*Camponotus* spp.: Hymenoptera: Formicidae) by Pheromones. Physiol Zool. 44(2): 77-83.

Bailey, I., Myatt, JP, Wilson, AM. 2013. Group hunting within the Carnivora: physiological, cognitive and environmental influences on strategy and cooperation. Behav Ecol Sociobiol 67: 1-17.

Bayliss, J, Fielding, A. 2002. Termitophagous foraging by *Pachycondyla analis* (Formicidae, ponerinae) in a Tanzanian coastal dry forest. Sociobiol. 39: 103-121.

Boesch, C. 1994. Cooperative Hunting in Wild Chimpanzees. Animal Behav 48: 653-667.

Brand, J, Mpuru, S. 1993. Dufour's gland and poison gland chemistry of the myrmicine ant, *Messor capensis* (Mayr). J Chem Ecol. 19: 1315-1321.

Bruckner, A, Hoenle, PO, von Beeren, C. 2018. Comparative chemical analysis of army ant mandibular gland volatiles (Formicidae: Dorylinae). Peerj 6.

Carroll, MJ, Schmelz, EA, Meagher, RL,Teal, PE. 2006. Attraction of *Spodoptera frugiperda* larvae to volatiles from herbivore-damaged maize seedlings. J Chem Ecol. 32: 1911-1924.

Cavill, G, Williams, P. 1967. Constituents of Dufour's gland in *Myrmecia gulosa*. J Ins Physiol. 13: 1097-1103.

Crewe, RM, Peeters, CP, Villet, M. 1984. Frequency-distribution of worker sizes in Megaponera foetens (Fabricius). S Afr Jor Zool. 19: 247-248.

Dechmann, DKN, Heucke, SL, Giuggioli, L, Safi, K, Voigt, CC, Wikelski, M. 2009. Experimental evidence for group hunting via eavesdropping in echolocating bats. Proc Biol Sci 276: 2721-2728.

D'Ettorre, P, Heinze, J. 2005. Individual recognition in ant queens. Cur Biol 15: 2170-2174.

Frank, ET, Linsenmair, KE. 2017a. Individual versus collective decision making: optimal foraging in the group-hunting termite specialist *Megaponera analis*. Animal Behav. 130: 27-35.

Frank, ET, Linsenmair, KE. 2017b. Flexible task allocation and raid organization in the termite-hunting ant *Megaponera analis*. Insectes Sociaux. 64: 579-589.

Frank, ET, Schmitt, T, Hovestadt, T, Mitesser, T, Stiegler, J, Linsenmair, KE. 2017. Saving the injured: Rescue behavior in the termite hunting ant *Megaponera analis*. Science Advances 3: e1602187.

Frank, ET, Wehrhahn, M, Linsenmair, KE. 2018. Wound treatment and selective help in a termite-hunting ant. Proc R Soc B. 285: 20172457.

Fujiwara-Tsujii, N, Yamagata, N, Takeda, T, Mizunami, M, Yamaoka, R. 2006. Behavioral responses to the alarm pheromone of the ant *Camponotus obscuripes* (Hymenoptera: Formicidae). Zool Sci. 23: 353-358.

Glancey, BM, Dickens, JC. 1988. Behavioral and electrophysiological studies with live larvae and larval rinses of the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). J Chem Ecol. 14: 463-473.

Guerrieri, FJ., Nehring, V, Jørgensen, CG, Nielsen, J, Galizia, CG, d'Ettorre, P. 2009. Ants recognize foes and not friends. Proc Biol Sci, 276(1666): 2461-1860.

Hölldobler, B, Braun, U, Gronenberg, W, Kirchner, WH, Peeters, C. 1994. Trail communication in the ant *Megaponera foetens* (Fabr.)(Formicidae, Ponerinae). J Ins Physiol. 40: 585-593.

Hölldobler, B, Wilson, EO. 1990. The Ants. Heidelberg, Berlin, Springer Verlag.

Holway, DA, Lach, L, Suarez, AV, Tsutsui, ND, Case, TJ. 2002. The causes and consequences of ant invasions. Annual Rev Ecol and Systematics. 33(1):181-233.

Janssen, E, Bestman, HJ, Holldobler, B, Kern, F. 1995. N, N-Dimethyluracil and Actinidine, two pheromones of the Ponerine ant *Megaponera foetens* (Fab.) (Hymoneptera: Formicidae). J Chem Ecol. 21: 1947-1955.

Lang, SDJ, Farine, DR. 2017. A multidimensional framework for studying social predation strategies. Nat Ecol Evol 1(9): 1230-1239.

Lepage, M. 1981. Ětude de la Predation de *Megaponera foetens* (F.) Sur lés populations recoltantes de Macrotermitinae dans un ecosysteme Semi-aridé (Kajiado- Kenya). Insectes Sociaux. 28: 247-262.

Lévieux, J. 1966. Noté préliminaire sur les colonnes de chasse de *Megaponera foetens* F. (Hymenoptera: Formicidae). Insectes Sociaux 13: 117-126.

Longhurst, C, Baker, R, Howse, P. 1979. Termite predation by *Megaponera foetens* (FAB.)(Hymenoptera: Formicidae). J Chem Ecol. 5: 703-719.

Longhurst, C, Johnson, R, Wood, T. 1978. Predation by *Megaponera foetens* (Fabr.)(Hymenoptera: Formicidae) on termites in the Nigerian southern Guinea savanna. Oecologia. 32: 101-107.

Lonnstedt, OM, Ferrari, MCO, Chivers, DP. 2014. Lionfish predators use flared fin displays to initiate cooperative hunting. Biol Lett 10(6): 20140281.

Schmidt, CA, Shattuck, SO. 2014. The Higher Classification of the Ant Subfamily Ponerinae (Hymenoptera: Formicidae), with a Review of Ponerine Ecology and Behavior. Zootaxa. 3817: 1-247.

Verheggen, FJ, Haubruge, E, Mescher, MC. 2010. Alarm Pheromones—Chemical Signalling in Response to Danger. Vitamins & Hormones 83: 215-239.

Villet, MH. 1990. Division-of-Labor in the Matabele Ant *Megaponera foetens* (Fabr) (Hymenoptera-Formicidae). Ethol Ecol Evol. 2: 397-417.

Wehner, R, Cheng, K, Cruse, H. 2014. Visual Navigation Strategies in Insects: Lessons from Desert Ants. New Visual Neurosciences, 1153-1163.

Yusuf, AA, Crewe, RM, Pirk, CWW. 2013. An effective method for maintaining the African termite-raiding ant *Pachycondyla analis* in the Laboratory. Afr Ento. 21: 132-136.

Yusuf, AA, Crewe, RM., Pirk, CWW. 2014b. Olfactory detection of prey by the termite-raiding ant *Pachycondyla analis*. J Insect Sci. 14(53)

Yusuf, AA, Gordon, I, Crewe, RM, Pirk, CWW. 2014a. Prey choice and raiding behaviour of the Ponerine ant *Pachycondyla analis* (Hymenoptera: Formicidae). J Nat Hist 48: 345-358.

Yusuf, AA, Pirk, CWW, Crewe, RM, Njagi, PGN, Gordon, I, Torto, B. 2010. Nestmate Recognition and the Role of Cuticular Hydrocarbons in the African Termite Raiding Ant *Pachycondyla analis*. J Chem Ecol 36: 795-796.