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**Protein profiling by MS shows evidence of royal protein  
pheromones in the cuticle extracts of the termite  
Reticulitermes flavipes**

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## Abstract

### Introduction

Proteins are known to work as social interaction signals in many species in the animal kingdom [1]. While they are common mediators of behavior in mammals and aquatic species, proteins have seldom been identified as such in insects. In social insects in particular, individuals have numerous physical contacts, providing many opportunities for the exchange of contact molecules as interaction mediators. However, their role in social interactions is largely unexplored: are they rare or simply underestimated? We set out to find out using molecular profiling of cuticular extracts from different castes and sexes of the termite *Reticulitermes flavipes*, which can be correlated with behavioral effects in these termites.

### Methods

Cuticle extracts of workers and reproductive individuals were obtained by washing live termites in water. Influence of cuticular extracts on workers behavior was assessed by video recordings. Molecular profiling of cuticular extracts was performed by MALDI-TOF MS using the ultra-thin layer spotting method [2]. Identification of proteins was carried out by top-down MS sequencing using MALDI-TOF/LIFT and ESI-QTOF CID fragmentation.

### Results

Using MS/MS methods, we show that aqueous cuticle extracts from both reproductive and worker termites contain proteins and/or peptides. Molecular profiling of protein from cuticular extracts using MALDI-TOF mass spectrometry reveals higher complexity in reproductives than in workers and a sex-specific composition exclusive to reproductives. Reproductives extracts impact workers body-shaking, a vibratory behavior involved in reproductives recognition, while extracts from workers do not. Thus, the difference in protein composition of cuticle extracts may underlie the specific effect of reproductives' extracts on workers behavior. Further MS characterization is necessary to identify the specific protein(s) responsible for this effect.

### Conclusions

These results indicate that social signaling may not be limited to cuticular hydrocarbons or other volatile chemicals and that proteins could play an important role as pheromones in insect societies. It also opens new perspectives to understand the role of proteins in reproductives' replacement, fertility signaling, or interspecific insect communication.

## Aqueous cuticle extracts contain proteins

### Top-down MS sequencing

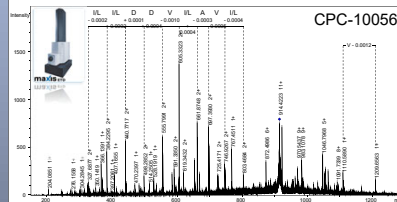


Fig. 5. Fragmentation mass spectrum of the CPC-10056 using UHR-ESI-QTOF-MS/MS CID. The precursor ion  $m/z$  914.4 (11+) is represented with a blue lozenge.

### Database search

Table 1: Eukaryota hits identified with the sequence tag ILILDDVILAVIL. The Peptide Search tool from Uniprot was used to interrogate the SwissProt and TrEMBL databases. Isoleucine and Leucine are treated as equivalent by Peptide Search.

Entry	Protein Name	Taxon
A0A371HC81	Protein GIGANTEA	Vridiplantae / Mucuna pruriens
A0A1US2M93	ADSL_C domain-containing protein	Fungi / Aspergillus nidulans
Q5B619	ADSL_C domain-containing protein	Fungi / Aspergillus nidulans
A0ASE3WGF4	RNA-directed RNA polymerase	Fungi / Peniophora
A0A6L2P7Q1	Uncharacterized protein	Metazoa / Insecta / Termitidae / Coptoterme
A0A6L2PEY0	Uncharacterized protein	Metazoa / Insecta / Termitidae / Coptoterme
B0W6D5	G12	Metazoa / Insecta / Culex

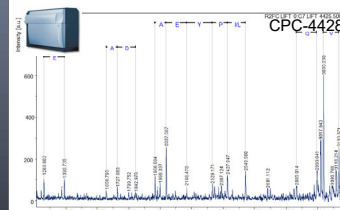


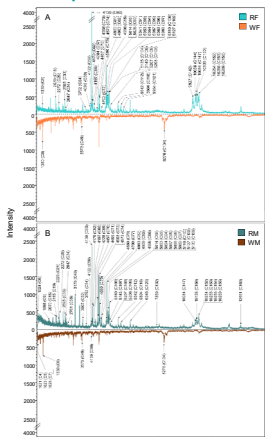
Fig. 6. Fragmentation mass spectrum of the CPC-4428 using MALDI-TOF/LIFT. The precursor ion is  $m/z$  4428 (1+).

Table 2: Results of protein- and nucleotide- sequence based TDS MASCOT search in TermiDB and NCBI databases. All matches with Mascot score lower than 53 were considered insignificant.

Compounds	Average m/z [M+H] <sup>+</sup>	Precursor m/z	MS/MS method	Protein database hit	Nucleotide database hit
CPC-4428	4427.7	4425	1 LIFT	No significant match	No significant match
CPC-10056	10055.8	914.4	11 CID	No significant match	JK369675.1 from Coptoterme formosus metatranscriptomic cDNA library, score = 81

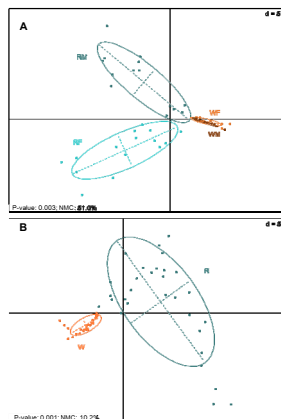
## MALDI-TOF MS profiling

### Reproductives extracts are more complex than workers extracts



197 CPCs (cuticular protein compounds) ranging from  $m/z$  1 000 to 25 000

### PLS-DA analyses based on the relative areas of CPCs discriminate extracts according to their caste and sex



87 discriminants CPCs

Fig. 1: Averaged mass spectra of cuticular aqueous extracts 675 from MALDI-TOF analyses. Spectra are shown in positive/negative display for reproductives versus workers for both (A) females and (B) males. The spectra are based on a sum of 15 spectra for each caste and sex. Annotated peaks are the first-quarter of the 87 major CPCs identified as discriminant by the PLS-DA analyses with VIP values greater than 1. Note that the 3 major CPCs 4428, 10056 and 10196 are also represented in the figure to support the discussion even if they are not in the first-quarter.

Fig. 2: PLS-DA plot based on the means of the areas of the 197 CPCs identified from cuticular aqueous extracts ( $n=15$  per group; peaks were selected using the Sum algorithm with a  $S/N \geq 4$  and a peak width of 5 Da). (A) Score plot of female reproductives (RF, light turquoise), male reproductives (RM, dark green), female workers (WF, light orange), and male workers (WM, dark orange). (B) Score plot of reproductives (dark green) and workers (light orange). Score plot display centroids and dispersion ellipses (cellipse=1.5).

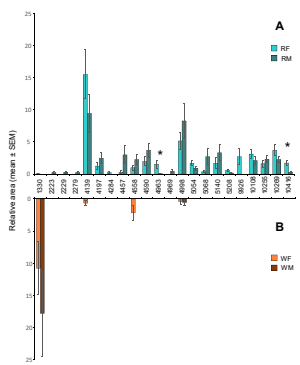


Fig. 3: Relative areas of the first-quarter of the 87 major CPCs identified as discriminant by PLS-DA analyses for reproductives (A) and workers (B) of both sexes: female reproductive (RF), male reproductives (RM), female workers (WF) and male workers (MW). Relative areas are represented as mean ( $\pm$  SEM) and are calculated on all the spectra. \* indicates significant differences (Wilcoxon signed-rank test,  $p < 0.05$ ; Multiple comparisons were corrected with FDR as the p-value adjustment method).

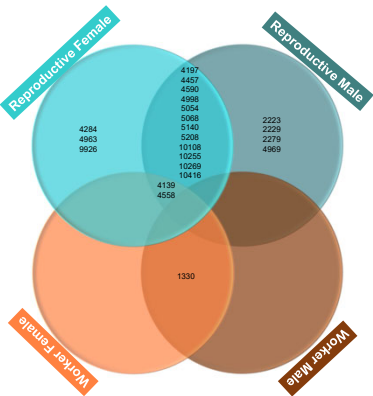


Fig. 4: Compounds repartition across castes and sexes of the first-quarter of the 87 major CPCs. CPCs were attributed to a group if more than one individual present the compound.

## Reproductives extracts impact workers behavior

### Reproductives extracts impact workers body-shaking, a vibratory behavior involved in reproductives recognition, while extracts from workers do not

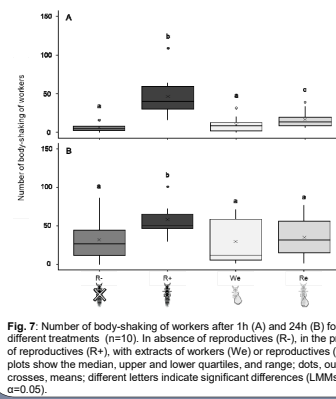


Fig. 7: Number of body-shaking of workers after 1h (A) and 24h (B) for the different treatments ( $n=10$ ). In absence of reproductives (R-), in the presence of reproductives (R+), with extracts of workers (We) or reproductives (Re). Box plots show the median, upper and lower quartiles, and range; dots, outliers; crosses, means; different letters indicate significant differences (LMWS,  $\alpha=0.05$ ).

### Body-shaking vary over time

Time dependent comparisons showed an increase of body-shaking events at 24h compared to 1h in all treatments, except for reproductives extracts where it was only marginally significant ( $Z = 8.5$ ,  $p = 0.059$ )

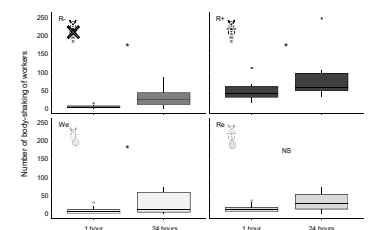


Fig. 8: Number of body-shaking of workers after 1h and 24h for the different treatments ( $n=10$ ). In absence of reproductives (R-), in the presence of reproductives (R+), with extracts of workers (We) or reproductives (Re). Box plots show the median, upper and lower quartiles, and range; dots, outliers; crosses, means; \* indicates significant differences (Wilcoxon signed-rank test,  $p < 0.05$ ).

## Discussion - Conclusion

Analyses of termite aqueous extracts of termite revealed a complex mixture of CPCs, which was caste and sex dependent. Mass spectrometry fragmentation confirmed that the profiled compounds are indeed proteins, with at least one example of an ortholog of G12, a digestive protein in termites. CPCs composition was more complex in reproductives than workers, with sex-specific CPCs for both male and female reproductives. This diversity of CPCs, with sex and caste specific compositions, could encode complex communication signals like the cuticular hydrocarbons (CHCs) which are well known to be involved in several social interactions.

Cuticular aqueous extracts from reproductives were enough to trigger body-shaking, while extracts from workers did not. Time dependent comparisons showed an increase of body-shaking events at 24h compared to 1h in all treatments, except for reproductives extracts where it was only marginally significant. This general increase, for both the mean values and the data variances, may reflect a behavioral instability due to social isolation. Indeed, tested micro-nests are fragments of larger mother colonies displaying rich interactions. Therefore, workers might be more behaviorally disturbed after 24h of isolation than after 1h. At 24h, the presence of reproductives still induce body-shaking but the reproductives extracts do not anymore. This short effect of the protein extracts might be due to a lack of stability overtime.

The combination of protein profiling with behavioral studies represents a powerful tool to explore unknown aspects of insect communications, which could help to answer unresolved questions about social communication.

Future studies will be needed to sequence and identify reproductive-specific CPCs and to test their behavioral functions, either individually or in synergy, to determine their role in the regulation of social interactions in insects, as has been already observed in vertebrates or aquatic organisms.

### Acknowledgments

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### References

- [1] Wyatt TD. (2014) Anim. Behav. 97, 273-280
- [2] Cadene M. and Chait B.T. (2000) Anal Chem. 72, 5655-5658