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FELLOWSHIP FINAL REPORT

Exploiting the Poultry Red Mite chemosensation for improvement of its control with entomopathogenic fungi

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REPORT INFO

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ABSTRACT

The Poultry Red Mite is considered the number one arthropod enemy of the poultry industry; despite this, this pest is hardly studied in terms of alternative control. The enormous economic loss they caused worldwide and the inefficacy of its control urge for solid solutions. One of the reasons why Dermanyssus gallinae is so challenging to manage is its hiding nature. Indeed, no treatment can reach them efficiently in their hiding spots. The idea of this project is to study the chemical ecology of the mite to attract it out of its hideouts. Le Studium Foundation has been instrumental in bringing together Prof Patricia Golo, an entomopathogenic-fungi specialist, and Dr. Fotini Koutroumpa, a specialist on arthropod chemosensation. Here, we would like to report preliminary results for optimizing the mite olfactory behavior tests in the laboratory and the first attempt to study one chemosensory gene expression of the mite. Comparison of these assays between fungus-treated and untreated mites was the goal of the project to understand if the entomopathogenic fungi could change the mite chemosensation and, therefore, negatively impact the fungus treatment. Further, we aimed to understand if a generally considered attractive molecule could enhance mite control with the fungus by masking the fungus's eventually repulsive molecules or/and attracting the mite to the fungus source. This collaboration set the base for further exchange to attend to these goals.

1- Introduction

The poultry red mite *Dermanyssus gallinae* is the most common ectoparasite on poultry and causes high economic losses in poultry farming worldwide [1]. These blood-feeding ectoparasites primarily target chickens but can also affect other poultry species. Infestations lead to reduced egg production, decreased growth rates, and increased bird stress, resulting in financial losses for poultry farmers and the industry.

Red Poultry Mites can cause skin irritation, anemia, and in severe cases, even death [2]. Ensuring the well-being of farmed animals is an essential aspect of animal husbandry, and effective control of these mites is crucial to maintaining optimal poultry health and welfare in France.

Conventional chemical control methods often involve the use of potent pesticides, which can have adverse effects on the environment and non-target organisms. Besides that, The Red Poultry Mite has developed resistance to several chemical pesticides commonly used for its

control [3]. This poses a significant challenge for farmers and pest management experts in finding effective control measures. Exploring alternative and sustainable methods, such as the use of entomopathogenic fungi, becomes crucial in combating mite resistance and reducing pesticide use.

The sensory system of arthropods plays a crucial role in their survival, behavior, and ability to find hosts for feeding [4]. Most knowledge of the sensory system of arthropods comes from insects. Although it is known that the sensory system of mites is composed of various specialized organs that allow them to perceive and interact with their environment, detailed knowledge of *D. gallinae* sensory system is missing in the literature. This is even truer for the interactions between this mite and entomopathogenic fungi in the context of sensory exchange and induced behavior. Unveiling these positives or negatives interactions is a step forward to achieving the full potential of biological control using entomopathogenic fungi.

Our project aimed to assess the virulence of different entomopathogenic fungal isolates and discover if the entomopathogenic infection, on its first days, has any impact on the mites' behavior. Here we also tried to optimize the Whole-mount RNA fluorescent *in situ* hybridization (WMFISH) technique of mite's legs and propodossoma, which has never been performed before. WMFISH is a powerful molecular technique to visualize specific RNA molecules' spatial distribution and expression patterns within intact biological samples [5]. This method allows researchers to detect genes' expression in one organism and specifically associate it to a tissue. Here we wanted to target chemosensory-related genes to unveil their expression in the legs of *D. gallinae* where the olfactory organ is present. Our goal would be to test if treatment with entomopathogenic fungi could alter the expected expression pattern of such genes in the mite legs.

2- Experimental details

Mites

Mites were collected from naturally infested French poultry farms in the region of the Val De Loire. Only mites from farms where no synthetic acaricides have been applied were used.

Entomopathogenic fungi

Four *Metarhizium* spp. isolates were used in the present study: *Metarhizium robertsii* isolates ARSEF 23 and ARSEF 2575, *Metarhizium brunneum* ARSEF 1095, and the commercial product LALGUARD M52® (Lallemand). ARSEF fungal isolates were obtained from the Agriculture Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF) (USDA-US Plant, Soil and Nutrition Laboratory, Ithaca, NY, USA). Conidia for the experiments were grown on PDAY (potato dextrose agar plus 0.01% yeast extract) at 25°C for 14 days. Conidia were harvest by scraping using a bacterial loop and suspended in 0.01% Tween 80 in 15-mL centrifuge tubes and vigorously agitated (vortexed). Conidial viability was measured by placing a 50 mL drop of fungal suspension on a PDAY plate and germination was observed by compound microscope (400×) after 24 hours at 28°C.

Fungal virulence assays

Bioassays were performed with *D. gallinae*. Conidia of each isolate were used to prepare fungal suspensions. Conidial concentrations were estimated by hemocytometer counts and adjusted to 1×10^8 conidia mL^{-1}. Only conidial suspensions with at least 95% germination were used. 55-mm-diameter Petri plates were modified to allow liquid evaporation from applied suspensions. A square hole (30×30 mm) was made in each lid of the Petri plates. The hole was closed with filter paper (40×40 mm) place internally in the plate, glued on the edges of the plate's lid. Filter paper was impregnated with 0.25 mL of sterile water containing 0.01% Tween 80 without (control) or with fungal conidia. Adult mites (30–40 per dish) were distributed on the impregnated filter paper with a brush. The mites were counted and incubated at 25 °C and 80% relative humidity (RH). The numbers of dead mites were counted daily under a stereomicroscope. Each treatment was

replicated three times for each isolate and the whole bioassay was repeated at least two times.

Dermanyssus gallinae behavioral experiments

Behavioral experiments with fungus-treated mites

The most virulent fungal isolate was chosen for the behavioral assays. *D. gallinae* response (attraction/avoidance) to 0.15% NH_3 was analyzed with previously *M. brunneum*-ARSEF-1095 treated mites or untreated mites. Mites were treated with fungi as previously described. Live mites were used in the behavioral assays three days after the treatment. Choice tests took place in glass Petri plates. The set-up consisted of one Petri plate (90×15 mm) with two filter-paper discs (diameter 10 mm each) (**Figure 1**). Each disc received 10µL of 0.15% NH_3 (test compound) or sterile ultrapure water (control) and was left to dry for 30 minutes. Only one mite was placed in the center of each plate to avoid aggregation behavior. The mite could choose between the test compound and the control or stayed in the neutral area (**Figure 1**). The experimental arena consisted in four Petri plates arranged in a square outline on a LED light rectangular pad. The LED light was used to improve contrast and allow the mite tracking. Videos of 10 minutes were recorded with an iPhone 12 camera to evaluate the mite's behavior. Sixty plates (15 recorded videos with 4 plates in each) were blindly analyzed using a semi-automated macro on the ImageJ software (version1.54f; https://imagej.nih.gov/ij/index.html). (30 plates with treated mites and 30 with untreated mites). Mites positioned on the lateral plate wall were not considered for the analysis.

The different areas considered are shown in **Figure 1**. The darkest areas show the treated disk surface (=on paper) in orange for the tested molecule and in blue for the solvent (here water). Two other areas with lighter colors show areas around the treated disk where diffusion of the tested molecule occurs strong enough to trigger behavior. In other words, mites on all three colored areas in **Figure 1** are considered under influence of the molecule on the respective disk and therefore attracted. Mites in the neutral area were considered not attracted. The mites' positions were tracked every four minutes. Mites' behaviors were assessed according to the workflow presented in **Figure 2**. Briefly, the videos (.mov format) were converted to .tif format using Photoshop software (CS6 version 13.0; Adobe, San José, CA, United States). This conversion reduced the size of the files analyzed by selecting one image every four minutes. The images were then analyzed using ImageJ software (version 1.54f; https://imagej.nih.gov/ij/index.html). A semi-automated macro was developed. This was used to isolate individual plates, determine the edges of the arena and the position of the two paper discs. Mites were then identified by thresholding after subtraction from the rest of the image. The position of the mite was then tracked frame by frame and verified manually. Data were analyzed by Two-way ANOVA followed by Sidak post hoc test.

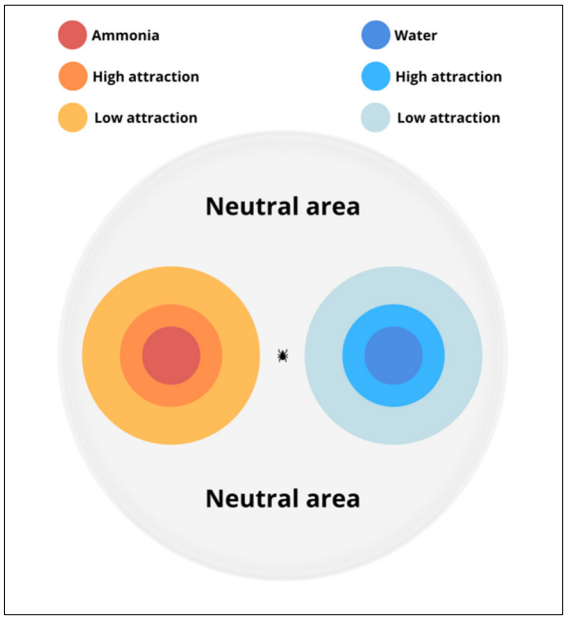

Figure 1: Schematic representation of the two-choice test. The mite under test is placed on the center of the Petri plate.

Behavioral experiments with clean (untreated) mite

Fungus uninfected mites were submitted to a two-choice test in the glass Petri plates set up as described before (**Figure 1**). In the first assay,

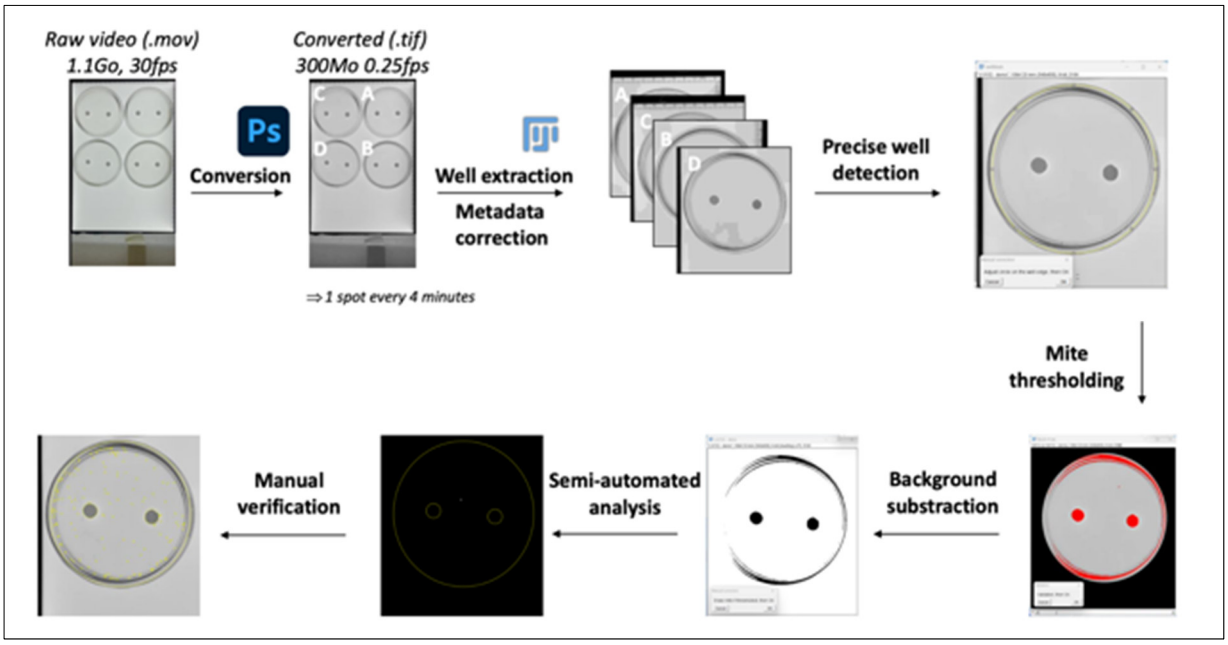

Figure 2: Schematic representation of the workflow for the detection of the mite position in the arena.

five different compounds were tested (i.e., Ultrapure sterile water, Tween 80 0.01% aqueous solution, fungal suspension, chicken blood, and chicken blood + fungal suspension). *M. brunneum* ARSEF 1095 was used to prepare the fungal suspension (10^8 conidia mL^{-1}). The mites could choose between 1) Pure chicken blood or ultrapure sterile water (12 recorded videos with four plates in each = 48 mites tested); 2) Pure chicken blood or 0.01% Tween 80 aqueous solution (six recorded videos with four plates in each = 24 mites tested); 3) Fungal suspension or 0.01% Tween 80 (eight recorded videos with four plates in each = 32 mites tested); or 4) Fungal suspension or chicken blood + fungal suspension (three recorded videos with four plates in each = 12 mites tested). In the second assay four different compounds were tested (i.e., ultrapure water, blood, and 1.5% NH_3). The mites could choose between 1) 1.5% NH_3 or ultrapure sterile water (6 recorded videos with four plates in each = 24 mites tested); 2) Pure chicken blood or ultrapure water (six recorded videos with four plates in each = 24 mites tested). Each disc received 10µL of the test compound and was left to dry for 10 minutes. Only one mite was placed in the center of each plate to avoid influence on the expected behavior due to aggregative behavior. Analysis of mites' behavior (=percent of time spent in each area) will be done as previously described.

RNA *In situ* hybridization of whole-mount mite's legs and propodossoma

Fungus-untreated adult mites were used in this experiment. The legs and the propodossoma of the mites were dissected and incubated in chitinase-chymotrypsin dimethyl sulfoxide buffer (CCD) according to the literature [6,7]. The permeabilization was optimized using DAPI staining (D9542-Sigma). After this step of permeabilization the tissue was fixed in paraformaldehyde 4% followed by prehybridization, hybridization and fluorescent staining according to Fleischer et al. [8].

Both digoxigenin and biotin probes were prepared for the same sequence fragment of the ionotropic receptor co-receptor IR25a. The probes used for the hybridization were designed using GenBank published sequence for *D. gallinae* with annotation number GIFZ01007959.1, from transcriptome shotgun assembly. A fragment of 1,556 base pairs was amplified and reverse transcribed using the Roche SP6/T7 transcription kit.

3- Results and discussion

Efficacy of different strains of *Metarhizium* spp. against adult mites

All *Metarhizium* spp. ARSEF isolates were virulent to *D. gallinae* adults (**Figure 3**) with survival percentages ranging from to 58 ± 12% (*M. robertsii* ARSEF 2575, 8 days after the treatment) to 50 ± 15% (*M. brunneum* ARSEF 1095, 8 days after the treatment). The commercial product MET 52 exhibited very little acaricidal efficacy against the mite's population that was tested. The average mites' survival rate yielded by MET 52 was 91 ± 4% and it was not different from the control group (untreated mites) ($P = 0.59$), five days after the treatment. Our result does not concur with Tomer et al. [9] where mites treated with MET 52 exhibited less than 40% survival, 5 days after the treatment. This dissimilarity between the two studies could be population dependent. Another explanation could be the Petri plates used in the experiments. Here, we modified the Petri plates (please refer to material and methods) to allow liquid evaporation. This resulted in much higher survival of control mites than in other studies, confirming the importance of reducing the mites' contact with liquid.

Fungus-treated mites' behavioral responses to NH_3

No repellent or attractive responses were observed in the behavioral tests with the fungus-treated mites.

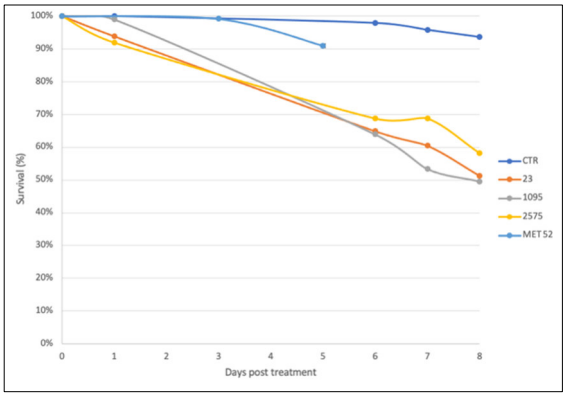

Figure 3: Survival curve of a French population of *Dermanyssus gallinae* treated with different entomopathogenic fungal isolates. (**CTR**) Tween 0.01% aqueous solution; (**23**) *Metarhizium robertsii* ARSEF 23; (**2575**) *M. robertsii* ARSEF 2575; (**1095**) *M. brunneum* ARSEF 1095; (**MET 52**) Commercial product based on conidia of *M. anisopliae*.

No significant difference was observed in the attraction behavior of the fungus-treated mites compared to the behavior of water-treated mites (control) **(Figure 4).**

According to Masier et al. [10], different populations of *D. gallinae* were reported to have different attraction profiles to NH3. Besides that, our analysis tracked the mites' positions every four minutes. This time is suggested to be too long for tracking the mites, which walk continuously and fast in the Petri plate, masking the results. We are now developing methods to shorten the mites' tracking interval in our videos.

Untreated mites' behavioral responses to NH_3, blood or *Metarhizium brunneum*

These results are yet to be analyzed.

RNA *In situ* Hybridization of Whole-Mount Mite's Legs and Propodossoma

The DAPI proved to be very useful for optimizing the tissue permeabilization step.

Five and a half hours were necessary for full permeabilization of the propodossoma up to the tip of the tarsi, where the olfactory organ is based. Three hours were enough for fully permeabilizing individually dissected legs (**Figure 5**). However, no staining with either digoxigenin or biotin was detected. One hypothesis is that the probes used were too big to penetrate the tissue or that a higher concentration is needed. Both hypotheses will be evaluated soon, using a higher concentration of the full-length probes used before and the preparation of new, shorter probes to be tested.

4- Conclusion

Different entomopathogenic fungal isolates have different virulence profiles towards the Red Poultry Mite *D. gallinae*.

The commercial product based on *M. anisopliae* tested against our local *D. gallinae* population has very low acaricidal effect.

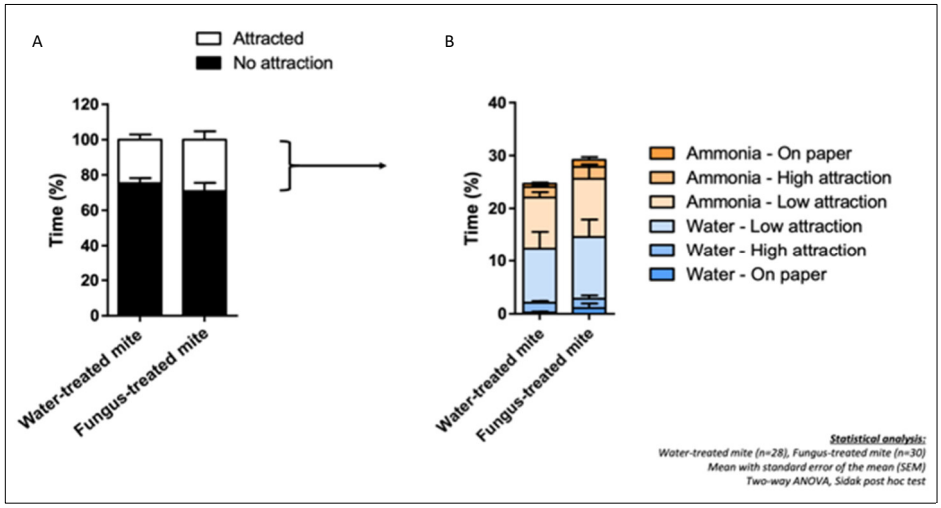

Figure 4: Time spent by the mite in or outside the attraction areas in the two-choice test. (**A**) Time spent in the attractions areas (water or NH_3) or outside the attraction areas (=no attraction/neutral area). (**B**) Time spent in the attraction areas separated by the different zones (on the paper, high attraction, low attraction – please refer to Figure 1).

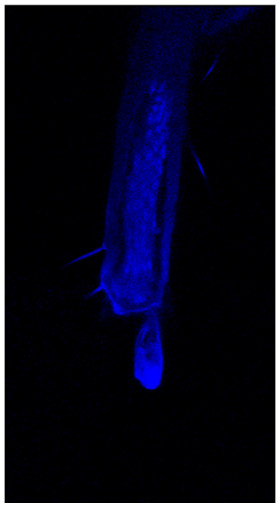

Figure 5: Posterior region of the first leg of a *Dermanyssus gallinae* mite. Cells' nuclei are stained with DAPI (blue).

The *D. gallinae* population tested here does not exhibit attraction to 0.15% NH_3, contrary to our initial hypothesis.

All cells' nuclei were detected under the legs (olfactory organ) of *D. gallinae*. Comparison of DAPI stained nuclei location, the sensory sensilla above them, and future staining provided by specific probes will give us insights into certain sensilla functions in olfaction or gustation.

5- Perspectives of future collaborations with the host laboratory

Dr. Patrícia Golo and Dr. Foteini Koutroumpa are organizing an online event on September 13 and 14, 2023 sponsored by Le Studium entitled: "*Brazil × France scientific partnership opportunities and alternative strategies for arthropod pest control*". The scientific scope of this event includes, but is not limited to, the following: international scientific collaborations, funding agencies that support Brazil × France scientific partnership, bioinputs, entomopathogenic fungal-arthropod host interactions, host-pathogen chemical interactions, reverse chemical ecology applications, and drug resistance.

The collaboration between Prof. Patrícia Golo and Dr. Fotini Koutroumpa on D. gallinae will continue with each researcher working on their sides on Brazilian and French populations, respectively. Other exchanges will be scheduled in the future for result comparison and application on both populations.

A future collaboration is on the verge of establishing between Prof. Patrícia Golo, Dr. Fotini Koutroumpa and the professors Claudio Lazzari and Fernando Guerrieri from the Institut de Recherche sur la Biologie de l'Insecte, CNRS-Université de Tours. The aim of this collaboration is to access the behavior of mosquito larvae when exposed to entomopathogenic fungal suspension.

6- Articles published in the framework of the fellowship

No articles were published in the framework of the fellowship.

7- Acknowledgements

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Biologie de l'Insecte, CNRS-Université de Tours. We also thank Fabrice Guegnard (INRAE-MPN team), David Pasdeloup (INRAE-BIOVA team) and Veronique Le Noach (private) for providing us with mites.

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