Trans-generational immunisation in the acrobat ant *Crematogaster scutellaris*

Adele Bordoni1\*, Leonardo Dapporto1, Irene tatini1, Martina Celli1, Manuel Bercigli1, Serena Resurreción Barrufet1, Brunella Perito1, Stefano Turillazzi1.

1Biology Department, Florence University, 50019 Sesto Fiorentino, Italy.

Supplementary methods and results

*M. anisopliae* spore suspension and growth conditions

To verify the virulence of *Metarhizium anisopliae* commercial spores (Met52© Monsanto) against ants, we rinsed a series of ten foundresses of *C. scutellaris* in a suspension of the commercial product. After the death of the foundresses, we collected the fungal spores by washing the ant bodies showing growth of mycelium in Triton solution (0.01% in distilled water). The conidiospore suspension was plated on Maltose Extract Agar (MEA, OXOID) in Petri dishes and incubated at 30°C for some days. Conidia from individual colonies were recognized as belonging to *M. anisopliae* under microscope according to their morphology and then collected in Triton solution. Dilutions of spore suspension were plated on MEA and incubated at 30°C. The number of - Colony-Forming Units (CFU) were counted to determine the spore viable title (CFU/ml).

Determination of the Lethal dosage for foundresses and workers

To assess the lethal dosages of *M. anisopliae* conidia on foundresses, we prepared suspensions with different conidia concentrations (3×103, 3×104, 3×105,3×106, 3×107 CFU/ml), and a control Triton solution, and then rinsed ten foundresses for 3 seconds in each suspension.

Ants were reared in the darkness in a thermostatic room (20°C-25°C temperature, 80% humidity). To facilitate the germination of the spores, the foundresses were kept at 100% humidity during the first two days (cit). After 14 days, we evaluated the mortality of the foundresses revealing the following values:

Triton solution = 0%

3×103 CFU/ml = 0%

3×104 CFU/ml = 20%

3×105 CFU/ml = 10%

3×106 CFU/ml = 20%

3×107 CFU/ml = 50%

Although the concentration of 3×104 CFU/ml resulted in a mortality of 20%, the comparison of the other values suggested that 3×105 CFU/ml can be considered a reliable LD10, and thus it was used as the low-dosage to expose foundresses.

On January 30th we treated 20 foundresses with Triton solution (0.01%) and allowed them to found colonies in spring conditions (darkness, 25°C-18°C temperature, 80% humidity). In each colony, at the emergence of the first worker, we added a plastic petri dish (5cm diameter) where we supplied *ad libitum* water, sugar and chopped dry dog food. We covered the queen chamber with aluminium foil to maintain darkness, while we exposed the foraging chamber to a 12-12h LD cycle. On May 23rd we collected 30 workers and we assessed the *Metarhizium* lethal dosage by rinsing ten workers in each of the following spore suspensions:

3×103 CFU/ml = 20%

3×104 CFU/ml = 80%

3×105 CFU/ml = 90%

We used the 3×104 CFU/ml as LD80 suspension for the challenges.

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| --- | --- | --- | --- | --- | --- |
| Treatment | n | Dead claustral  | Live claustral  | Producing suitable workers | mean workers ± s.d. |
| Triton | 35 | 6 | 3 | 20 | 3.2±1.7 |
| Naïve | 35 | 6 | 2 | 15 | 2.5±1.1 |
| Mt spores | 35 | 4 | 2 | 16 | 2.8±1.1 |
| Dead Mt spores | 32\* | 8 | 3 | 18 | 4.3±2.4 |

Table S1. Number of queens used in the study for each treatment (n), queens dead before day 150 without producing the first worker (Dear claustral), live queens at day 150 which did not produce the first worker (Live claustral), queens which produced workers suitable for the challenge (at least two worker produced with a difference in adult age of less than 10 days, see methods), mean and standard deviation of workers used for the challenges for each treatment. In the dead Mt spores groups, three queens died in the day of the treatment and they have been removed from the analysis.



Figure S1 Survival plot for the workers subjected to the two protocols (feeding and starvation) during the 14 day challenge with a DL80 dose of *M. anisopliae*. In parentheses number of colony having produced suitable workers and total number of workers tested for each protocol.