Title: Infection signaling and antimicrobial wound care in an ant society

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Abstract: Infected wounds pose a major mortality risk in animals. Injuries are common in the ant *Megaponera analis* which raids pugnacious prey. Here we show that *M. analis* can determine when wounds are infected and treat them specifically. By applying a variety of antimicrobial compounds and proteins secreted from the metapleural gland to infected wounds, workers reduce the mortality of infected individuals by 90%. Chemical analyses showed that wound infection is associated with specific changes in the cuticular hydrocarbon profile thereby likely allowing nestmates to diagnose the infection state of injured individuals and apply the appropriate antimicrobial treatment. This study demonstrates that the targeted use of antimicrobials to treat infected wounds, previously thought to be a uniquely human behavior, has evolved in insect societies as well.

One-Sentence Summary: Ants use antimicrobial compounds and proteins to successfully treat the infected wounds of nestmates.

Main Text: Infected wounds are a major mortality risk for animals (*1, 2*), but the identification and medicinal treatment of infected wounds is thus far considered a uniquely human behavior. While several mammals have been shown to lick wounds and apply saliva (*1, 2*), the efficacy of this behavior remains largely unknown and occurs indiscriminately of the state of the wound. Workers of the predatory ant *Megaponera analis* have been shown to care for the injuries of nestmates *(3, 4)* which are common because this ant feeds exclusively on pugnacious termite species. As many as 22% of the foragers engaging in raids attacking termites have one or two missing legs (*3*). Injured workers are carried back to the nest where other workers treat their wounds (*4*). When the wounds of injured workers are not treated by nestmates 90% of the injured workers die within 24 hours after injury (*4*), however the mechanisms behind these treatments are unknown.

To investigate whether the high mortality of injured individuals is due to infection by pathogens, we collected soil from the natural environment and applied it to the wounds of experimentally injured workers (i.e., a sterile cut in the middle of the femur on the hind leg of an otherwise healthy ant). After 2 hours, injured ants exposed to the soil (hereafter referred to as "infected ants") had ten times higher bacterial loads in the thorax than similarly injured individuals exposed to sterile phosphate buffered saline (PBS, hereafter referred to as "sterile ants"; Wilcoxon test: W=0; P<0.001; Fig. 1A, table S1). After 11 hours, bacterial load further increased in infected ants (Wilcoxon test: W=4; P<0.001), while there was no such increase in sterile ants (Wilcoxon test: W=41; P=0.5; Fig. 1A). As a result, after 11 hours the bacterial load was 100 times higher in infected than sterile ants (Wilcoxon test: W=10; P=0.009; Fig. 1A). A microbiome analysis further revealed major differences in bacterial species composition and abundance between the two groups of ants (ADONIS: F=17.45; R^2=0.31; P<0.001; Fig. 1B & S1A), with three potentially pathogenic bacterial genera increasing in absolute abundance in the thorax of infected ants at both time-points: *Klebsiella*, *Pseudomonas* and *Burkholderia* (fig. S1B). These differences in bacterial composition and abundance between infected and sterile ants were associated with important differences in survival probability, with survival being seven times lower for infected ants (least square means: Z=-4.246; P<0.001; Fig. 1C, S2A & table S2).

To test if wound care by nestmates could reduce the mortality of infected ants, we either placed infected and sterile ants in their colony or kept them in isolation. The mortality of infected ants kept with their nestmates was much lower than the mortality of infected ants kept in isolation (least square means: Z=-2.759; P=0.02; Fig. 1C). By contrast, there was no significant difference between the mortality of sterile ants kept with their nestmates or in

isolation (least square means: Z=1.04; P=0.89; Fig. 1C). The mortality of infected ants was also not significantly different than the mortality of sterile ants when these individuals were kept with their nestmates (least square means: Z=-0.630; P=1; Fig. 1C). Overall, these data demonstrate that *M. analis* workers are capable of effectively treating wounds that have been exposed to soil pathogens.

By culturing the soil medium on agar plates, we were able to isolate two potential pathogens (the endosymbiotic bacterium *Burkholderia* sp. and its fungal host *Rhizopus microsporus*, and the bacterium *Pseudomonas aeruginosa*, Fig. 1B, S1CD). While the application of *B.* sp. and *R. microsporus* (separately or together) on wounds did not significantly decrease survival (fig. S2B & S3), the application of *P. aeruginosa,* a bacterium widespread in various environments (*5*), caused a 95% mortality within 36 hours (Fig. 2A). Since the treatment with only *P. aeruginosa* was as deadly as the treatment with all soil pathogens (fig. S3), we only used *P. aeruginosa* in subsequent infection assays to better control pathogen load.

Similar to the experiments where the soil was applied to the wound, the presence of nestmates was also effective in decreasing the mortality of injured workers exposed to a known concentration of *P. aeruginosa* (OD=0.05). While the mortality of infected ants kept in isolation was 95%, mortality of infected ants that had been returned to their nestmates was only 10% (least square means: Z=-2.94; P=0.01; Fig. 2*A,* S2C & table S3). There were major differences in the increase in *Pseudomonas* load after injury between ants kept with or without their nestmates (Fig. 2B & table S4). The bacterial load of infected ants kept with their nestmates did not increase significantly from two and 11 hours after injury (least square means: t=-0.037, P=1; Fig. 2B). By contrast, there was a 100-fold increase in bacterial load for infected ants kept in isolation (least square means: t=-4.832, P<0.001; Fig. 2B).

To study the proximate reason for the reduced mortality of infected ants when they are returned to their nestmates, we introduced injured ants (with sterile and infected wounds) to their nestmates and filmed them for 24 hours. We observed that workers treated the injury of infected ants by depositing secretions produced by the metapleural gland (MG) which is located at the back of the thorax (Fig. 3A). The MG secretions, which have antimicrobial properties (*6-10*), were applied in 10.5% of the wound care interactions (43 out of 411). Before applying MG secretions, the nursing ant always groomed the wound first (i.e., "licking" the wound with their mouthparts). Nursing ants then collected the secretions either from their own MGs (movie S1), as described in other species

(*8*), or from the MG of the injured ant itself (movie S2). Wound care with MG secretions lasted significantly longer (85±53s) than wound care without MG secretions (53±36s; Wilcoxon test: W=12468, P<0.001; fig. S4). Remarkably, workers were able to discriminate between infected and sterile ants. Wound care treatment was provided more often to infected ants (ANOVA: Treatment: F=6.9, P=0.01; Fig. 3B). Moreover, MG secretions were deposited significantly more often on wounds of infected than sterile ants (ANOVA, wound type: F=4.7, P=0.03), in particular between 10 and 12 hours after infection (Fig. 3C).

Because cuticular hydrocarbons (CHCs) are known to be frequently used as a source of information in ants (*11*), we investigated whether infected ants could signal their injured state through changes in the profile. Immediately after injury, infected ants did not differ from sterile ants in their CHC profile (ADONIS: R^2=0.13; F=0.13; P=0.96; Fig. 4, table S5 & S6). This profile changed in both types of ants during the two hours after injury (Sterile ants: ADONIS: R^2=0.15; F=2.74; P=0.03; Infected ants: ADONIS: R^2=0.13; F=2.42; P=0.05; Fig. 4), converging towards a similar profile for both types of ants (ADONIS: R^2=0.008; F=0.17; P=0.87; Fig. 4). Thereafter, the CHC profile of infected ants remained unchanged until 11 hours after injury (ADONIS: R^2=0.063; F=1.48; P=0.13; Fig. 4), while the CHC profile of sterile ants changed significantly (ADONIS: R^2=0.14; F=3.54; P=0.04; Fig. 4) thereby becoming significantly different from the CHC profile of infected ants (ADONIS: R^2=0.19; F=5.4; P=0.007; Fig. 4).

Consistent with the idea that changes in CHC profile could provide information on the health status of ants (*12*), the observed differences in the CHC profiles (Fig. 4) mostly stemmed from differences in the relative abundance of alkadienes (fig. S5, table S7), which are among the CHC compounds most relevant for communication in social insects (*13*). These changes in the CHC profile are generally regulated by differentially expressed genes in the fat body (*14*). To identify the genes likely responsible for the observed CHC changes between infected and sterile ants we conducted transcriptomic analyses of the fat bodies of the same individuals. A total of 18 genes related to CHC synthesis were differentially expressed between infected and sterile ants 11 hours after exposure to *P. aeruginosa* (17 genes out of 378 that were differentially expressed were immune genes; fig. S6A, table S8 & S9), while only two genes related to CHC synthesis were differentially expressed two hours after infection (in addition to 7 immune genes out of 164; fig. S6B, table S8 & S9).

To quantify the capabilities of MG secretions to inhibit bacterial growth, we conducted antimicrobial assays. The growth of *P. aeruginosa* was reduced by >25% when MG secretions were included in a lysogeny broth (LB) solution compared to a control LB solution (Wilcoxon test: W=54, P<0.001, Fig. 3D).

Since *P. aeruginosa* has repeatedly developed antimicrobial resistance (*15*) and because most antimicrobial compounds found in animal saliva are unable to inhibit the growth of *P. aeruginosa* (*16*), we examined the content of the MG secretions, conducting proteomic and chemical analyses. The proteomic analysis revealed 41 proteins (fig. S7 & table S10), 15 of which showed molecular similarity to toxins, which often have antimicrobial properties (*17*). Five proteins had orthologs with functions involving antimicrobial activity (e.g., lysozyme, hemocytes, 2 MRJP1-like proteins) and three with melanization, a process implicated in wound healing in insects (*18, 19*). Nine proteins could not be attributed a clear function. These included the most abundant protein detected in the MG secretions (13±16% of the MG's endogenous protein content), a protein for which no ortholog could be found. The evolutionarily young gene coding this protein could be a promising candidate for antimicrobial or antibiotic research (*20*). The gas-chromatography/mass-spectrometry (GC-MS) analyses of the MG further revealed 112 organic compounds (23 of which could not be identified, fig. S8, table S11). Six of the identified compounds had antibiotic- and/or fungicide-like structures and 35 were alkaloids. While we could not identify the exact structure of the alkaloids, many of them are known to have antimicrobial properties (*21*). There were also 14 carboxylic acids, making up 52% of the secretions content (fig. S8, table S11), probably leading to a lower pH detrimental to bacterial survival and growth (*6*).

The diversity of chemical compounds identified in the MG's secretions of *M. analis*, 112, is far greater than those found in other ant species where the number of compounds ranges between 1 and 35 and mostly consists of carboxylic acids rather than alkaloids or antibiotic-like compounds (*6*). While the use of the MG secretions has been observed in other ant species to sterilize the nest or as a response to fungal exposure (*6, 8, 10*), it had never been observed in the context of wound care. The use of the MG secretions probably fulfills a similar role as the antiseptic saliva of mammals during wound care, both harboring functionally similar antimicrobial and wound healing proteins *(22)*, but in mammals this treatment has never been observed to depend on the infected state of the wound.

This study reveals a highly effective behavioral adaptation to identify and treat festering infections of open wounds in ants. The prophylactic and if necessary therapeutic use of antimicrobial secretions to counteract infection in *M. analis* (Fig. 3C) mirrors modern medical procedures for dirty wounds (*23).* Remarkably, the primary pathogen in ant's wounds, *Pseudomonas aeruginosa*, is also a leading cause of infection of combat wounds in humans, where infections can account for 45% of casualties (*24*). This demonstrates convergence in both the challenges of warfare and the solutions that evolved to mediate them across human and insect societies.

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Project administration: ETF, PE, LaK

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Writing – original draft: ETF, LaK

Writing – review & editing: ETF, LuK, JL, QH, ACL, AD, FA, EPE, PW, PE, TS, LaK

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Data and materials availability: Raw amplicon-sequence data was deposited at the Sequence Read Archive (SRA) under PRJNA826317. Sequence reads were deposited in NCBI Sequence Read Archive (SRA) under the accession number PRJNA823913.The proteomics data was deposited at the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD033003. The remaining data is available in the Dryad Digital Repository: https://doi.org/10.5061/dryad.hqbzkh1j6

Supplementary Materials

Materials and Methods

Figs. S1 to S8

Tables S1 to S11

References (*25*–*53*)

Movies S1 to S2

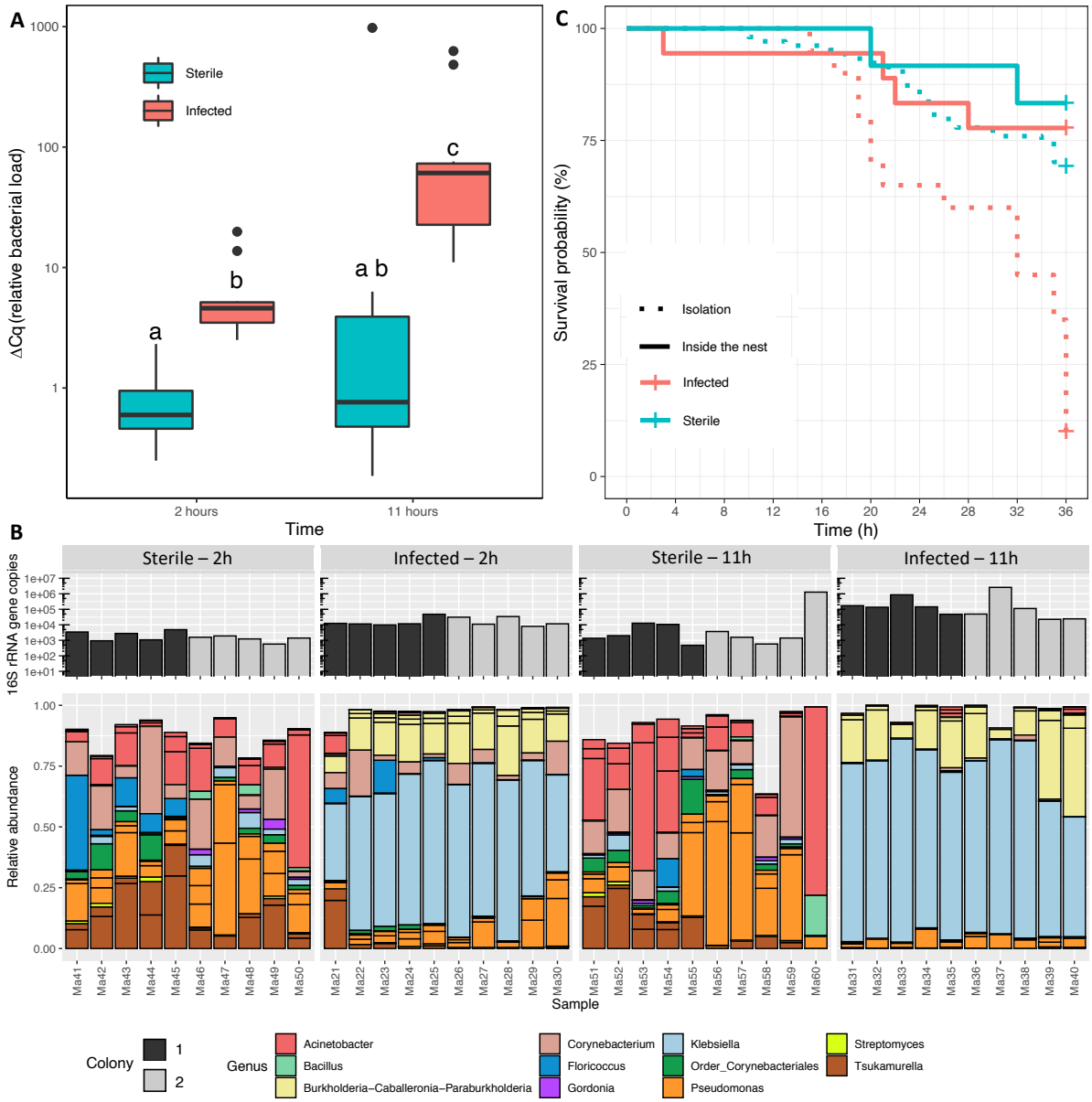

Fig. 1 Lethal effects and diversity of soil pathogens. (A) Relative 16S rRNA gene copies (bacterial load ΔCq) for individuals whose wounds were exposed to a sterile PBS solution (Sterile) or soil pathogens diluted in PBS (Infected, OD=0.1) for 2 and 11 hours after exposure (see table S1 for statistical results). *n*=10 per boxplot, significant differences (*P*<0.05) are shown with different letters. **(B)** 16S rRNA gene copy numbers and relative abundance of bacterial genera present in the thorax for the same individuals as in Fig. 1A. Multiple bars of the same color indicate different amplicon-sequence variants (ASVs) belonging to the same genera. ADONIS: Treatment: F=17.45; R^2=0.31; P<0.001 **(C)** Kaplan – Meier cumulative survival rates of workers in isolation (dotted line) or inside the nest (solid line) whose wounds were exposed the same way as in Fig. 1A (infected or sterile). Detailed statistical results in fig. S2A and table S2.

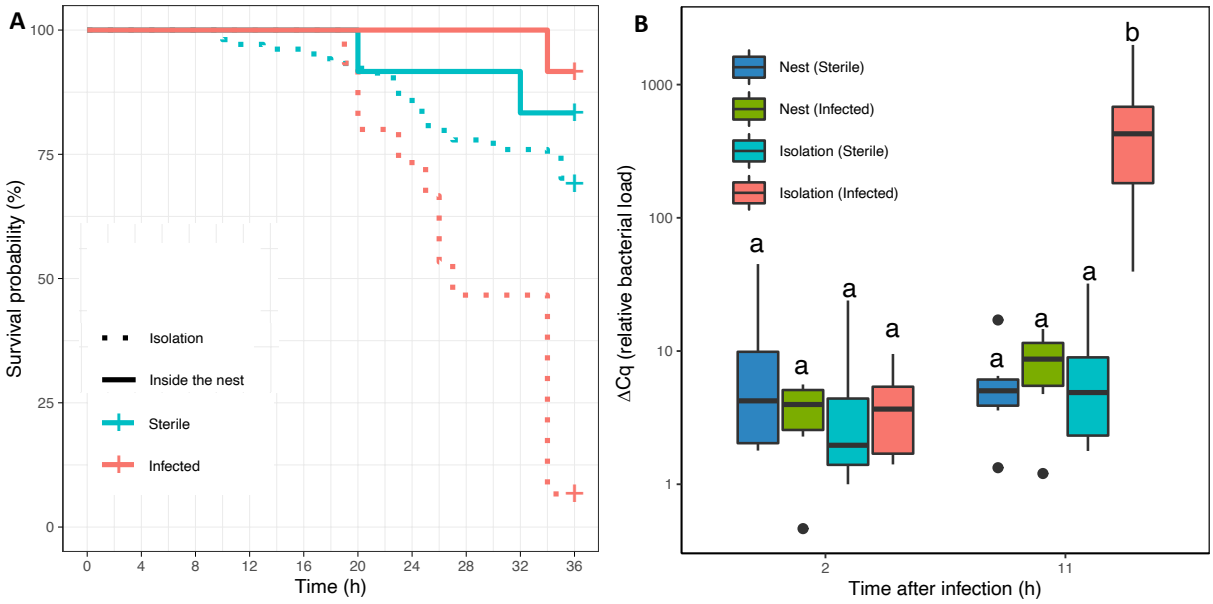

Fig. 2 Survival probability and pathogen load of sterile and infected ants. (A) Kaplan – Meier cumulative survival rates of workers in isolation (dotted line) or inside the nest (solid line) whose wounds were exposed to *P. aeruginosa* diluted in PBS (Infected, OD=0.05) or a sterile PBS solution (Sterile). Detailed statistical results in fig. S2C and table S3. **(B)** relative bacterial load (ΔCq) of *Pseudomonas* at two different time points (2h and 11h) for ants in isolation or inside the nest with wounds treated the same way as in Fig. 2A (Infected or Sterile). *n*=6 per boxplot, significant differences (*P*<0.05) are shown with different letters (table S4).

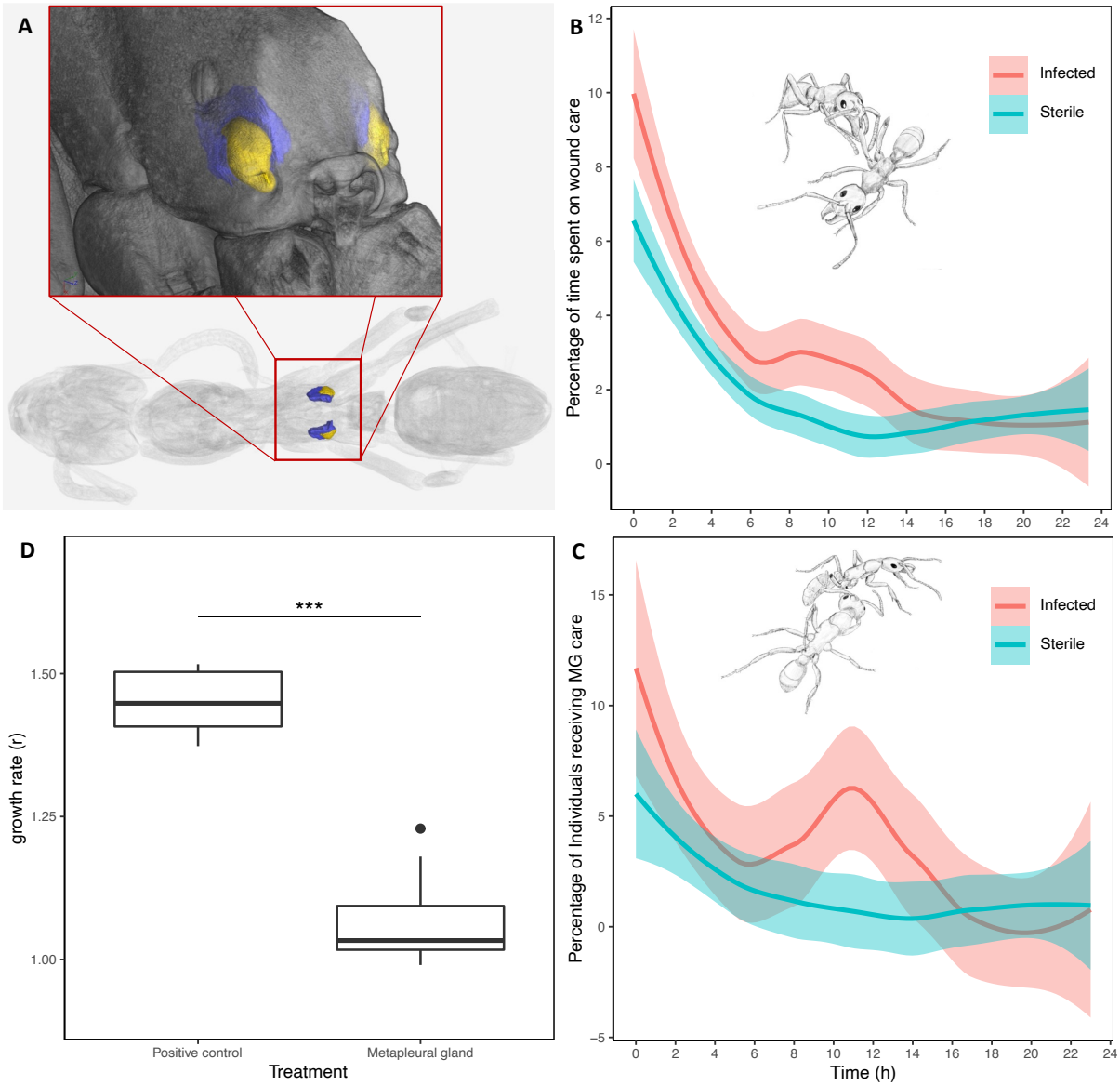

Fig. 3 Use and efficacy of the metapleural gland (MG) secretions during wound care. (A) Micro CT scan showing the location of the MG. Blue: secretory cells; yellow: atrium. **(B)** Percentage of time spent on wound care over 24 hours with a local polynomial regression (loess) showing a 95% confidence interval fitted for *n*=6 sterile ants (Sterile) and *n*=6 infected ants (Infected). LMER: formula= woundcare~time*treatment; Random effect (ID:Colony): Variance=25.66; Std.Dev.=5.065; Residual: Variance=1240.98; Std. Dev.=35.23; ANOVA: Time: *t*=87.7, *P*<0.001***, Treatment: *F*=6.9, *P*=0.013*; Time:Treatment: *F*=7.4 *P*=0.007**. **(C)** Percentage of individuals receiving wound care with MG secretions for the same ants as in Fig. 3B (Infected and Sterile) using the same loess model. LMER: formula= MGcare~time*treatment; Random effect (ID:Colony): Variance=0.49; Std.Dev.=0.70; Residual: Variance=54.2; Std. Dev.=7.36; ANOVA: Time: *F*=14.8, *P*<0.001***, Treatment: *F*=4.7, *P*=0.03*; Time:Treatment: *F*=2.2 *P*=0.13. **(D)** Bacterial growth assay for *P. aeruginosa* either in LB broth (positive control, *n*=6) or LB broth with MG secretions (Metapleural gland *n*=9). Wilcoxon test: *W*=54, *P*<0.001.

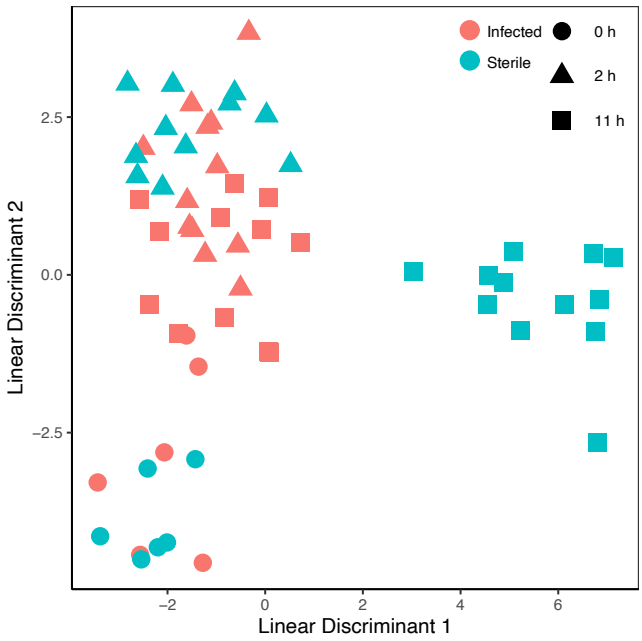

Fig. 4 Linear discriminant analysis of CHC profiles of sterile and infected (OD=0.05 of *P. aeruginosa*) ants at 0, 2 or 11 hours after manipulation. Detailed statistical results in table S5**.**