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Report

Ants Disinfect Fungus-Exposed Brood by Oral Uptake and Spread of Their Poison

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Summary

To fight infectious diseases, host immune defenses are employed at multiple levels. Sanitary behavior, such as pathogen avoidance and removal, acts as a first line of defense to prevent infection [1] before activation of the physiological immune system. Insect societies have evolved a wide range of collective hygiene measures and intensive health care toward pathogen-exposed group members [2]. One of the most common behaviors is allogrooming, in which nestmates remove infectious particles from the body surfaces of exposed individuals [3]. Here we show that, in invasive garden ants, grooming of fungus-exposed brood is effective beyond the sheer mechanical removal of fungal conidiospores; it also includes chemical disinfection through the application of poison produced by the ants themselves. Formic acid is the main active component of the poison. It inhibits fungal growth of conidiospores remaining on the brood surface after grooming and also those collected in the mouth of the grooming ant. This dual function is achieved by uptake of the poison droplet into the mouth through acidopore self-grooming and subsequent application onto the infectious brood via brood grooming. This extraordinary behavior extends the current understanding of grooming and the establishment of social immunity in insect societies.

Results and Discussion

Hygienic Brood Care Reduces Number and Viability of Fungal Conidiospores

We studied the sanitary behavior of workers of the invasive garden ant *Lasius neglectus* after exposure of their pupae to the entomopathogenic fungus *Metarhizium brunneum*. After 24 hr of brood care, during which the workers performed intensive brood grooming, we washed off the remaining fungal conidiospores from the pupal surface and determined their number and germination ability in comparison to a worker-absence control. As we expected from previous work [3], we found that brood care significantly reduced pathogen load on the pupae (mean ± SEM number of conidiospores per pupa washed off; worker absence: 24,320 ± 2,476, worker presence: 4409 ± 489; generalized linear model (GLM) with quasi-Poisson errors, $F_{1,18} = 35.71$, $p < 0.001$). Interestingly, the remaining conidiospores had a significantly reduced germination ability (65%–89% proportion germination inhibition in presence versus absence of workers; GLM with quasi-binomial errors, $F_{1,18} = 118.91$, $p < 0.001$), suggesting that chemical disinfection may complement mechanical pathogen removal in *L. neglectus*, thereby revealing a dual function of sanitary brood care in ants.

To uncover the underlying mechanisms, we interfered with the performance of grooming behavior and the function of several exocrine glands. We glued the mouthparts of workers to prevent brood grooming and application of potential antimicrobial secretions from, e.g., the mandibular gland [4]. We further sealed the openings of the metapleural gland (MPG), a unique gland in ants producing antimicrobials for antipathogen defense [5], and the acidopore, which is the joint opening of the poison gland, the Dufour gland, and the hindgut. The emitted poison and Dufour gland substances likely have their main function in antipredator defense and trail following, respectively [6, 7] but also show antimicrobial properties [8, 9], allowing for a secondary role in antipathogen defense [10]. We found that successful removal of conidiospores depended on intact mouthparts but was not affected by blockage of the MPG and the acidopore (Figure 1A; number of conidiospores removed within 24 hr of brood care after a recovery phase of 3–5 hr after ant treatment; GLM with quasi-Poisson errors: $F_{4,74} = 36.89$, $p < 0.001$; Tukey post hoc tests: $p < 0.05$ for all pairwise comparisons except ns for worker presence versus both MPG and acidopore blockage and ns for mouth blockage versus worker absence).

Remaining conidiospores on the pupal surface had a more than 50% reduced germination ability after tending by control workers compared to worker absence and mouth blocking, which did not differ from each other. MPG blockage did not affect the ants' ability to reduce fungal germination, whereas acidopore blockage significantly impaired their antifungal capacity (Figure 1B; GLM with quasibinomial errors: $F_{4,74} = 21.92$, $p < 0.001$; Tukey post hoc tests: $p < 0.05$ for all pairwise comparisons except ns for worker presence versus MPG blockage and ns for all pairwise comparisons between worker absence, mouth blockage, and acidopore blockage). Whereas MPG secretions likely play a major role in protection of nest members against pathogens, including *Metarhizium*, in fungus-growing ants [5, 9, 11], our findings document that *L. neglectus* releases antifungal compounds from the acidopore, suggesting activity of the poison droplet. To directly test the role of the poison, we gently poked the ants' abdomen (gaster), causing immediate release of the poison droplet. Such poison-depleted ants lost most of their antifungal effect (Figure 1C; GLM with quasibinomial errors: $F_{4,56} = 21.99$, $p < 0.001$; see Figure 4B; Tukey post hoc tests: $p < 0.05$ for all pairwise comparisons)—the little inhibitory capacity that remained suggests that our experimental depletion may not have been complete, because gland replenishment is a long process [12]. Poison-depleted and acidopore-blocked workers did not differ in their grooming behavior from control (sham-glued) ants (see Figure S1 available online; grooming frequency: GLM with quasi-Poisson errors,

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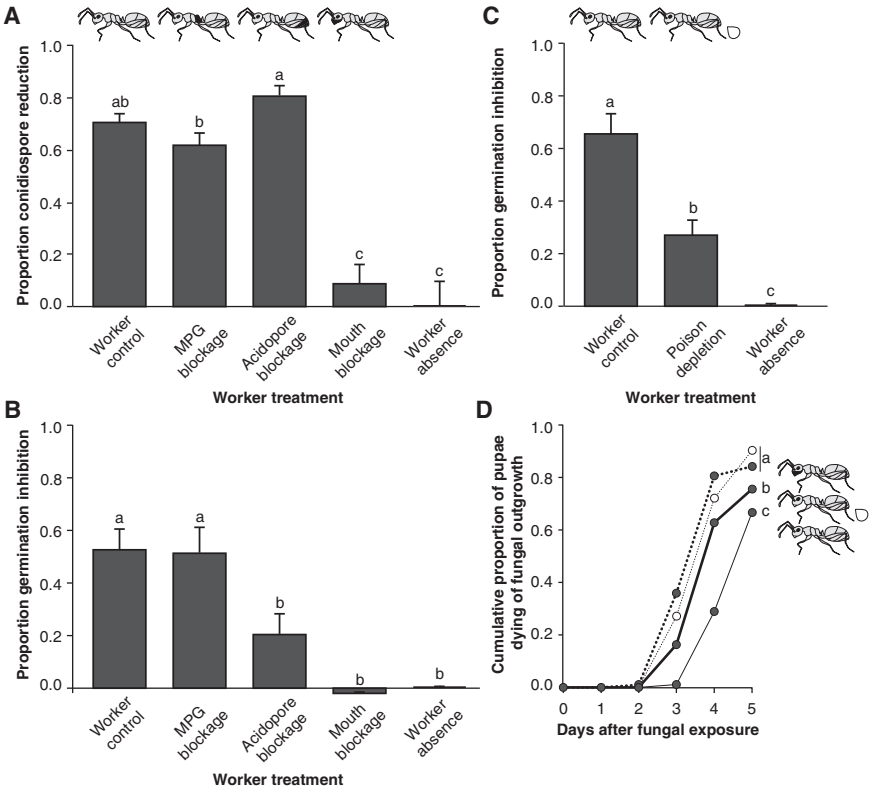

Figure 1. Effects of Brood Care on Fungal Conidiospore Number and Germination Ability, and Fungal Outgrowth from Pupae

(A) *L. neglectus* workers significantly reduced the number of conidiospores (*M. brunneum*) on the surface of fungus-exposed pupae during 24 hr of brood tending, compared to a worker-absence control. Removal of conidiospores requires active grooming and is impaired by mouth blockage but is not reduced by blockage of either the metapleural gland (MPG) or the acidopore.

(B) Workers also inhibited germination of conidiospores remaining on the surface of pupae, as revealed by germination checks of conidiospores washed off after 24 hr of tending and subsequently plated on agar. MPG-blocked workers inhibited fungal growth to the same extent as control workers. In contrast, blockage of the acidopore and the mouth prevented this antifungal effect.

(C) Poison-depleted ants also had a significantly reduced ability to inhibit fungal growth in comparison to control workers, but they still showed some antifungal effect compared to the worker-absence control.

(D) The cumulative proportion of pupae dying of fungal outgrowth within 5 days after exposure was highest in the absence of workers (open circles, fine dotted line) and when tended by mouth-blocked workers (filled circles, bold dotted line) that lack the ability to groom, intermediate for poison-depleted workers (filled circles, bold solid line) that can only mechanically remove conidiospores from the pupal surface, and lowest for control workers (filled circles, fine solid line) capable of both mechanical removal and chemical disinfection.

Bars in panels (A)–(C) show means + SEM. Different letters indicate statistically significant differences at $\alpha = 0.05$. For grooming behavior of the workers, see Figure S1.

$F_{2,33} = 0.46$, $p = 0.642$; grooming duration: GLM with quasi-Poisson errors, $F_{2,33} = 1.83$, $p = 0.195$), providing in vivo evidence for an antifungal activity of the poison droplet (Figures 1B and 1C).

The combination of mechanical removal and chemical disinfection during 24 hr of brood care decidedly affects the course of fungal infection. Pupae showed similarly fast and high fungal outgrowth when grooming was prevented by mouth blockage as in complete worker absence. Brood mortality was significantly reduced by poison-depleted workers that could only perform mechanical removal and even further decreased by control workers that were able to both remove and disinfect conidiospores (Figure 1D; Cox proportional regression: Wald test: $\chi^2 = 70.96$, df = 3, $p < 0.001$; Tukey post hoc tests: $p < 0.05$ for all pairwise comparisons except ns for worker absence versus mouth blockage).

Our findings on the protective in vivo poison use during brood care complement previous in vitro demonstrations of the antimicrobial activity of poison from other ants [8, 9, 13, 14] and wasps [15, 16] and observations that fire ants apply their poison onto their eggs during oviposition [17] and disperse it in the brood chamber by raising and vibrating their gaster ("gaster flagging" [18]). Self-produced poison was also found to play a protective role in individual immunity of some ants [9] and wasps and bees, which likely spread the poison over their body with their legs during self-grooming but do not transfer it to nestmates [19–21]. Further, some bees and wasps [16, 20]—but not other wasps [19] or ants [9]—seem to integrate their poison into the nest material, likely for nest sanitation. Together with our documentation of poison use for social immunity during brood care, this evokes a broad potential for antipathogen defense by poison in social insects.

Composition and Origin of the Substances in the Poison Droplet

Chemical analysis of the poison droplet by gas chromatography-mass spectrometry (GC-MS) revealed a total of 37 compounds (Figure S2A and Table S1). As expected from other formicine ants [22, 23], the large polar phase of the poison droplet contained mostly formic acid (concentration of 60%) and also acetic acid (2%) (Table S2; [23]). Both acids originate from the poison gland (confirmed by direct analysis of the poison gland reservoir, n = 10 replicates) and have described antimicrobial activity [8, 12], acting in vitro against *Metarhizium* fungus [9]. Four hydrocarbons from the Dufour gland [24] were the main substances of the smaller apolar phase (n-undecane, n-tridecane, 2-tridecanone, and 2-pentadecanone, 0.1%–0.6%; Tables S1 and S2), and they have no [25] or few [26] described antimicrobial effects. We also detected traces of longer chained fatty acids (C14, C16, C18), originating from the hindgut, which do not seem to have antimicrobial activity ([27]; B.M., unpublished data).

Poison Gland and Dufour Gland Substances Together Inhibit Fungal Germination In Vitro

To disentangle the relative contributions of the six major poison components, we determined their germination inhibition

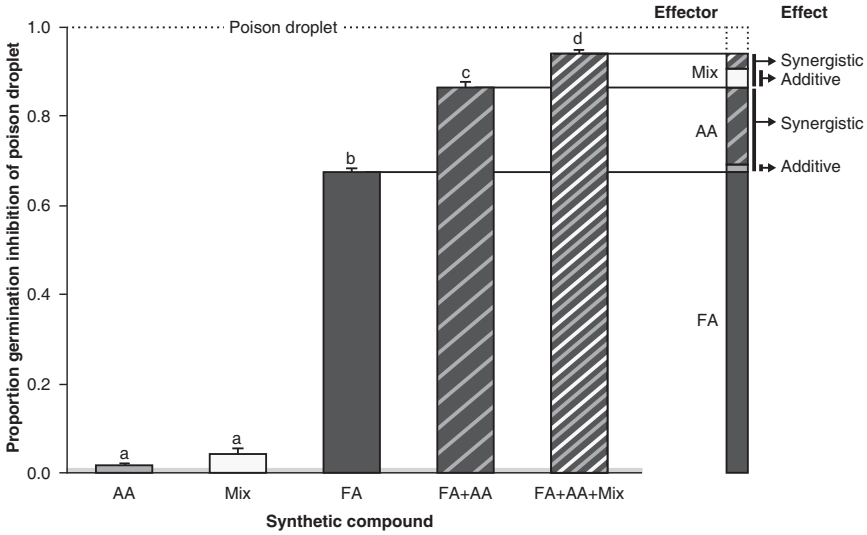

Figure 2. Contribution of the Chemical Components in the Ant Poison to Its Antifungal Effect

We determined conidiospore growth inhibition (*M. brunneum*) on agar plates after application of synthetic equivalents of the main components of the poison droplet in their natural concentrations (Figure S2A and Tables S1 and S2) to understand their contributions to the inhibitory effect found in the natural poison droplet of the ants. Acetic acid alone (AA, pale gray) did not significantly inhibit fungal growth, as illustrated by the gray shaded area above the x axis that depicts the 95% confidence interval of the conidiospore growth control. The mix of the four main Dufour gland substances ("Mix"; n-undecane, n-tridecane, 2-tridecanone, and 2-pentadecanone; white) had a weak but significant inhibitory effect on fungal growth compared to the conidiospore growth control (but not significantly different from AA). Formic acid (FA, dark gray) had a strong antifungal effect, alone explaining approximately 70% of the natural droplet inhibition. The inhibitory effect of formic acid increased synergistically by adding the second main poison gland substance, acetic acid (dark and pale gray stripes). Further addition of the Dufour gland mix (dark and pale gray plus white stripes) intensified the inhibitory effect even more, leading to a total inhibition of fungal growth and explaining 94% of the natural poison droplet. Bars display mean + SEM, with different letters indicating statistically significant differences at $\alpha = 0.05$. For gas chromatogram of poison droplet, see Figure S2A; for identification and glandular origin of poison droplet compounds, see Table S1; for quantification of the six main compounds in the droplet, see Table S2; and for effects of the individual Dufour gland compounds in the in vitro germination assay, see Table S3.

capacity both alone and in combination, using synthetic compounds (equivalent volume and concentration to natural droplet). The main effector of the antifungal activity was formic acid, which alone accounted for approximately 70% of the germination inhibition of natural poison droplets. Acetic acid did not show an antifungal effect on its own but enforced the activity of formic acid, revealing a synergistic action of the two compounds. The four hydrocarbons from the Dufour gland applied together had a low but significant antifungal effect, to which all individual components contributed similarly (Table S3). Addition of these Dufour gland compounds to formic and acetic acid further increased the inhibitory effect to a total of 94% of the natural ant poison droplet (Figure 2; Table S3; GLM with quasibinomial errors: $F_{5,35} = 815.01$, $p < 0.001$; Tukey post hoc tests: acetic acid versus conidiospore growth control, $p = 0.10$, and versus Dufour gland mix, $p = 0.07$; all other comparisons, $p < 0.01$). In contrast to explanations of the antimicrobial effect of poison from other ants [9] or MPG gland compounds [5], the inhibitory activity of the poison droplet of *L. neglectus* cannot simply be explained by its low pH of 2.5; other acids (hydrochloric and sulfuric acid) at the same pH did not inhibit fungal germination (mean ± SEM percentage of nongerminating conidiospores for hydrochloric acid: 3.1% ± 0.5%; sulfuric acid: 2.7% ± 0.4%; and conidiospore growth control: 3.0% ± 0.4%; GLM with binomial errors: $F_{2,17} = 0.46$, $p = 0.630$).

Although the main ancestral function of ant poisons may be protection against predators [6], they are known for their antimicrobial properties in vitro [8, 10], and social insects use them for, e.g., nest sanitation and as herbicide [10, 12, 28]. Birds and primates [29, 30] collect and sweep ants through their feathers or fur ("anting" behavior), likely to fight ectoparasites, and humans utilize formic and acetic acid for, e.g., food preservation [31] and livestock protection [32, 33]. It is therefore likely that our findings are not limited to *L. neglectus* ants fighting *Metarhizium* fungi, but that future work will demonstrate a broad-spectrum protection against a wide array of pathogens in colonies of formicine ants.

Constitutive and Induced Nature of Poison Application

Pupae in healthy colonies showed an acid cover on their surface that faded after 24 hr of isolation from workers, as visualized by a color change from blue to red, revealing acidity of pH < 4 when pupae were placed on pH-sensitive litmus paper (mean ± SEM, 0 hr: 0.033 ± 0.007; 24 hr: 0.007 ± 0.002; GLM with binomial errors: $F_{1,14} = 13.73$, $p < 0.001$; Movie S1). The acidic coverage was intensified by workers tending fungus-exposed, but not sham-treated, pupae over untreated controls (Figures 3A and S3A; GLM with quasibinomial errors: $F_{2,21} = 42.74$, $p < 0.001$; Tukey post hoc tests: fungus-exposed versus untreated or control-treated: $p < 0.001$; untreated versus control-treated: $p = 0.29$). In line with these acidity measures around pupae, we detected higher quantities of formic acid in the gas phase of nests in which workers tended fungus-exposed pupae compared to untreated or sham-treated pupae, which were indifferent (Figure 3B; GLM with gamma errors: $F_{2,59} = 9.37$, $p < 0.001$; Tukey post hoc tests: fungus-exposed versus untreated or control-treated: $p < 0.001$; untreated versus control-treated: $p = 0.91$). *L. neglectus* workers thus continuously apply a basal level of poison onto their healthy pupae and amplify this investment under pathogen pressure.

Direct versus Indirect Mode of Poison Application onto the Brood

Workers directly sprayed their poison onto the pupae by bending their gaster tip with the acidopore toward it (Figure 3C and Movie S1). This behavior was very rare, occurring only once every 27 hr when rearing sham-treated pupae and once every 4 hr after pupal fungus exposure (Figure 3C; GLM with Poisson errors: $F_{1,78} = 10.12$, $p = 0.001$). In addition, the ants performed frequent indirect poison application via a previously undescribed behavioral sequence of oral uptake of the poison by "acidopore grooming" and subsequent brood grooming (Movie S1). During acidopore grooming [34, 35], the workers self-groomed their acidopore by bending their head to the gaster tip "licking" the acidopore (Figure 3D). Acidic spillover

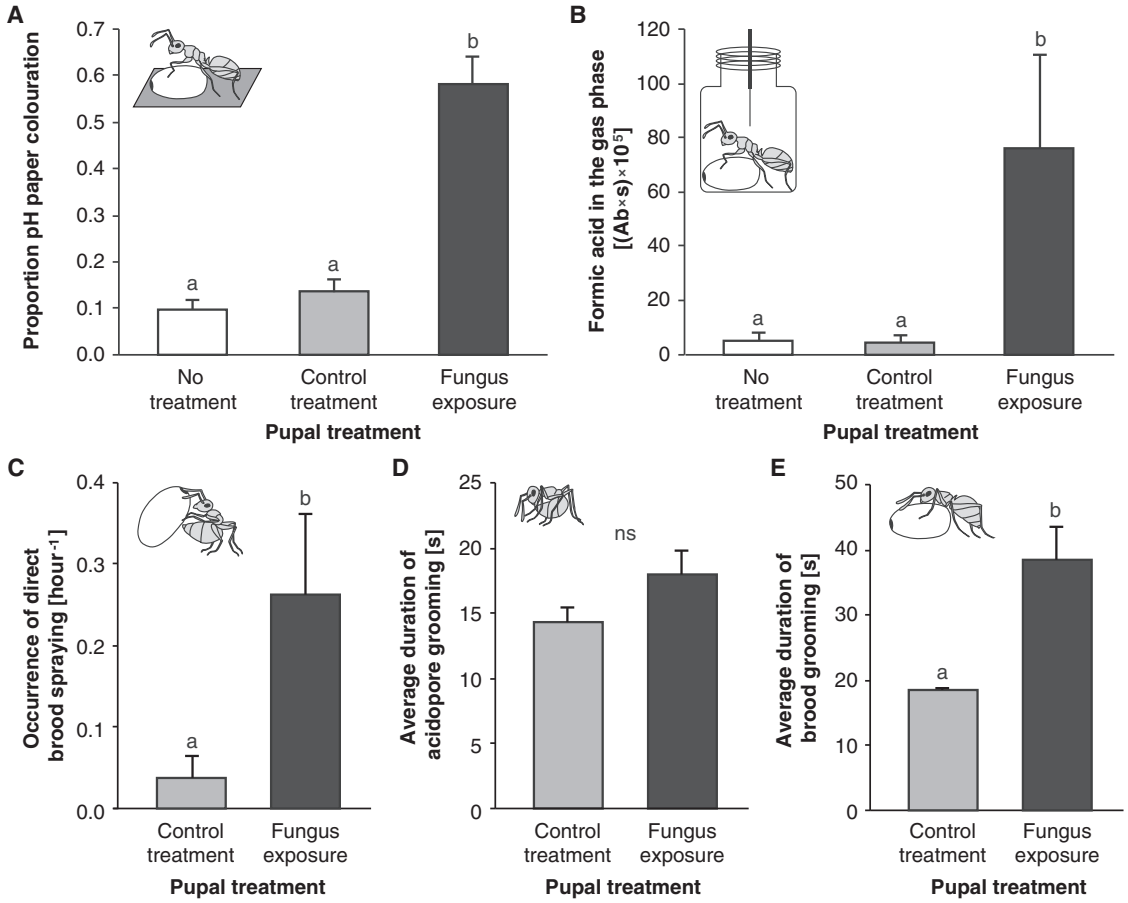

Figure 3. Pathogen-Induced Application of Poison onto the Brood

(A) Pupae were placed on pH-sensitive litmus paper, and the acidity of their surface after worker tending was determined by the area of color change from blue to red (indicating pH < 4). After 24 hr of brood tending by workers, the acidic coating of fungus-exposed pupae (*M. brunneum* conidiospores; dark gray) was significantly more intense as compared to both untreated and sham-treated pupae (white and pale gray, respectively; Figure S3A and Movie S1).

(B) The quantity of formic acid detected in the gas phase around tended pupae (SPME GC-MS; after 5 hr) was significantly higher for fungus-exposed pupae (dark gray) than for both untreated and sham-treated pupae (white and pale gray, respectively).

(C) Behavioral observations revealed that, in rare cases, *L. neglectus* workers applied the poison directly onto the brood by positioning the gaster tip close to brood surface and spraying the poison from the acidopore (Movie S1). This direct brood spraying was performed significantly more frequently toward fungus-exposed (*M. brunneum* conidiospores, dark gray) than sham-treated pupae (pale gray).

(D) Acidopore grooming, a behavior during which workers expel the poison droplet from their acidopore and take it up into their mouth (Movie S1), was not significantly higher in workers tending fungus-exposed versus sham-treated pupae.

(E) Workers groomed the pupae significantly longer (Movie S1) after fungus exposure (dark gray) than after sham-treatment (pale gray).

In all panels, bars show mean + SEM. Different letters indicate statistically significant differences at $\alpha = 0.05$; ns, nonsignificant. For visualization of pH paper color change, see Figure S3, and for performance of behaviors, see Movie S1.

detected on the pH-sensitive paper (Movie S1) suggested that, during this behavior, the poison droplet was emitted from the acidopore and taken up into the mouth. Whereas acidopore grooming duration did not significantly increase when workers tended fungus-exposed compared to sham-treated pupae (Figure 3D; GLM: $F_{1,27} = 2.38$, $p = 0.134$), pupal grooming was significantly prolonged under pathogenic conditions (Figure 3E; Movie S1; GLM: $F_{1,21} = 13.36$, $p = 0.001$) and occurred 50 times more frequently than direct spraying (Figures 3C and 3E). Indirect application of the antifungal droplet via the mouth thus seems to be the dominant mode of brood coating in *L. neglectus* ants.

Oral Poison Uptake

Chemical analysis of worker heads confirmed the presence of the three major poison compounds (formic acid, acetic acid, and n-undecane; Figure S2B and Table S4). Workers directly taken from the colony had much higher quantities of poison substances in their heads than workers with their acidopores blocked for 24 hr, whereas other compounds associated with the head and absent from the poison did not differ (see Table S4 for each compound and Figure 4A for formic acid). This provides direct proof for oral poison uptake and reveals that these volatile substances decrease in abundance when replenishment is precluded by acidopore blockage. Poison storage is effective for at least 1 hr, with no significant difference in formic acid quantity in heads of ants taken directly from the colony and with their acidopores blocked for 1 hr (Figure 4A; GLM: $F_{2,19} = 11.03$, $p < 0.01$; Tukey post hoc test, $p = 1.00$ for control workers versus 1 hr blockage; $p < 0.01$ for control or 1 hr versus 24 hr). The amount of poison in the head directly matched the ants' efficacy in inhibiting fungal

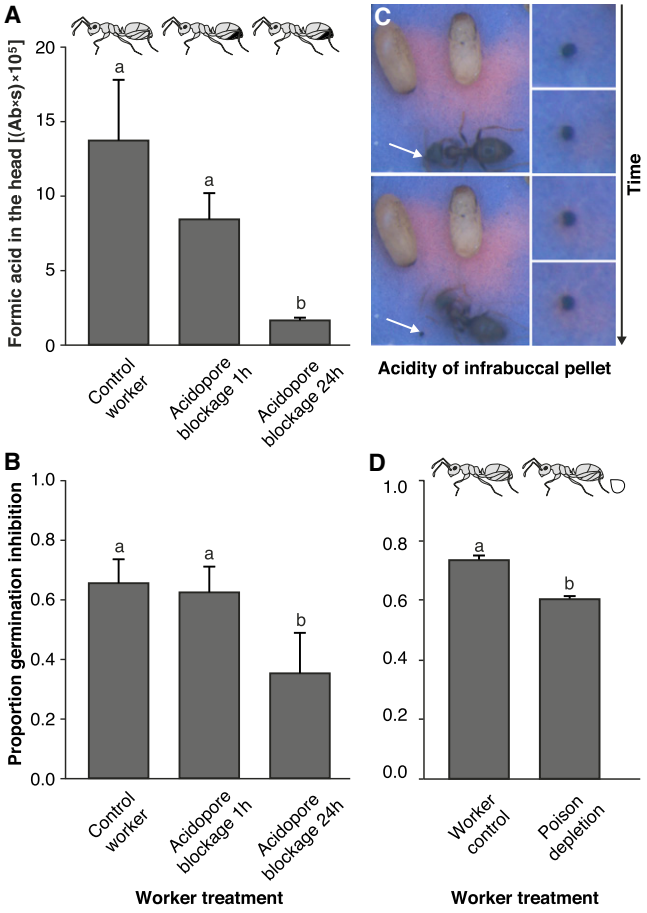

Figure 4. Poison Persistence in Worker Heads and Effect on Fungal Germination

(A) The quantity of formic acid detected in worker heads (Table S4 and Figure S2B) was equally high in ants collected directly from the colony (control workers) and those that had their acidopore blocked for 1 hr. In contrast, acidopore blockage for 24 hr led to a significant reduction of formic acid quantity, indicating that the poison is stored for at least 1 hr in the head.

(B) Acidopore blockage for 1 hr did not compromise the ability of workers to inhibit fungal growth, whereas 24 hr blockage led to a significant reduction of the antifungal effect. This suggests that the poison stored in the head keeps its potency for at least 1 hr.

(C) *L. neglectus* worker ant expelling an infrabuccal pellet (arrow) after grooming of fungus-exposed pupae (*M. brunneum* conidiospores, Movie S1; left: two large photographs). The bottom of the ant nest was covered with pH-sensitive litmus paper that changes from blue to red upon contact with the acid poison (indicating pH < 4). The red area around the pupae was caused by direct poison spraying (Movie S1). The pH-sensitive paper in the surrounding of the infrabuccal pellet changed color from blue to red within 23 s after expulsion of the pellet (right: four small photographs).

(D) Conidiospores were collected from infrabuccal pellets of poison-depleted versus control workers, and their germination on agar plates was determined. Poison depletion led to significantly lower antifungal activity.

In (A), (B), and (D), bars represent mean + SEM, and different letters indicate statistically significant differences at $\alpha = 0.05$. For chromatogram of worker heads, see Figure S2B; for compound identification and quantification, see Table S4; and for infrabuccal pellet expulsion, see Movie S1.

growth, so that only workers with their acidopore blocked for 1 hr, but not for 24 hr, showed similarly high inhibition levels as control workers (Figure 4B; GLM with quasibinomial errors: $F_{4,56} = 21.99$, $p < 0.001$; see Figure 1C; post hoc Tukey test, $p < 0.05$ for acidopore blockage 24 hr versus both control workers and acidopore blockage 1 hr, which are ns from each other).

Benefits of Oral Poison Uptake

Taking up poison into the mouth seems to be a risky strategy potentially causing self-damage. However, harm to the mouth chamber is probably small in ants due to its lining with relatively inert exoskeleton, so that this novel sanitary behavior has likely evolved due to selective advantages over direct spraying. We propose that application via grooming may allow higher accuracy (less spillage) than direct spraying, as well as a better distribution of the poison across brood items. Grooming workers frequently switch between pupae, whereas spraying involves application of a large amount on single pupae (Movie S1). Oral uptake may further reduce the risk for workers to contract infection during their sanitary brood care; they frequently self-groom their head and antennae directly after acidopore grooming (in 74% [14 of 19] of the observations; Movie S1). In addition, the poison has further function in disinfection of fungal conidiospores that the ants remove from the brood and collect in specific pockets in their mouth (infrabuccal pockets) before expelling them in form of an infrabuccal pellet [36]. Infrabuccal pellets are acidic (Figure 4C and Movie S1), and the germination rate of fungal conidiospores from these pellets was significantly lower when produced by control workers compared to poison-depleted ants (Figure 4D; GLM with quasibinomial errors; $F_{1,18} = 66.53$, $p < 0.001$). Oral poison uptake thus also reduces the fungal load in the nest environment.

Conclusion

Our study extends current knowledge on grooming leading not only to mechanical removal but also to chemical disinfection of pathogenic particles. Surface disinfection by self-produced antimicrobials is also known from other arthropods in the form of prey preservation [37] or the arthropod's own body protection [38, 39], and other social insects apply antimicrobials to themselves and their brood by leg movements (salivary gland compounds in termites [40, 41]; MPG substances in fungus-growing ants [11]). In all these cases, secretions are applied directly from the gland, in which they were produced and stored, to the site of action. It is unique, however, that *L. neglectus* ants transfer the poison from the site of production and first storage to a second storage site in the head. This could be due to an evolutionary extension in the use of the poison, from serving just as a defense against predators to also protecting against pathogens, thereby turning the mouth of workers into a "chemical disinfection chamber."

Experimental Procedures

We observed the behavior of workers of the ant *L. neglectus* during brood care of control and pathogen-exposed pupae (*M. brunneum*) and determined their capacity to mechanically remove and chemically inhibit the growth of the fungal conidiospores by counting and germination testing, respectively. Ants were manipulated by blockage of their mouthparts or exocrine gland openings or by poison depletion. GC-MS based on solid-phase microextraction (SPME) and liquid and solid injection was used for qualitative and quantitative characterization of compounds in the poison droplet, worker heads, and the gas phase around ants during performance of sanitary brood care. The antifungal effects of synthetic equivalents of the main poison compounds were determined in agar plate germination assays, and the application of poison onto the brood was quantified by the color change of pH-sensitive litmus paper. Expanded details of the study

system, experimental design, behavioral observations, chemical analysis, germination assays, and image and data analysis can be found in the Supplemental Experimental Procedures.

Accession Numbers

Raw data are available at DRYAD (http://datadryad.org/) under the DOI http://dx.doi.org/10.5061/dryad.61649.

Supplemental Information

Supplemental Information includes three figures, four tables, Supplemental Experimental Procedures and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2012.11.034.

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