

1**Title: Protection against the lethal side effects of social immunity in ants**

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3**Authors:** Christopher D. Pull^{1*,§}, Sina Metzler¹, Elisabeth Naderlinger¹, Sylvia

4Cremer^{1‡}

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6**Affiliations:**

71. IST Austria (Institute of Science and Technology Austria), Am Campus 1, 3400

8Klosterneuburg, Austria

9§ Present address: School of Biological Sciences, Royal Holloway University of

10London, Egham, Surrey, TW20 0EX, UK

11* Lead Contact and Corresponding Author: Christopher D. Pull

12(chris.pull@rhul.ac.uk)

13‡ Senior Author

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15**Key words**

16Disease defence, collective sanitation, antimicrobial compounds, prophylactic

17behaviour, immunopathology, social insects, cocoon, formic acid

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19**Main text**

20Many animals use antimicrobials to prevent or cure disease [1,2]. For example, certain
21plants are ingested to self-medicate when sick [2] and antimicrobial substances are
22used by animals as topical disinfectants, to prevent infection, protect offspring and to
23sanitise their surroundings [1,2]. Social insects (ants, bees, wasps and termites) have
24evolved collective disease defences to protect their colonies from epidemics that are
25functionally analogous to the immune system of individual organisms [3,4]. This
26“social immunity” utilises antimicrobials to prevent and eradicate infections, and to
27keep their brood and nest clean. However, these antimicrobial compounds can be
28harmful to the insects themselves, and it is unknown how colonies prevent collateral
29damage when using them. Here, we demonstrate that antimicrobial acids, produced by
30workers to disinfect the colony, are harmful to the delicate pupal brood stage, but that
31the pupae are protected from the acids by the presence of a silk cocoon.

33Formicine ants produce poison rich in formic and acetic acid – potent antimicrobials
 34also used by humans – from the poison gland at the tip of their abdomen, which they
 35use to sanitise pathogen-contaminated nestmates and brood [5 and refs therein], and to
 36kill and disinfect sick brood [6]. Here, we observed invasive garden ants (*Lasius*
 37*neglectus*) also using their poison prophylactically. When given new nest boxes, we
 38found that the ants regularly treated their surroundings with their acidic poison –
 39including the areas around the brood piles – over a two-day period, which we
 40visualised and quantified using pH sensitive paper (Figure 1A; likelihood ratio test
 41(LR)- $\chi^2 = 9.22$, $df = 1$, $P = 0.002$; post hoc results in figure). Ant brood is hence
 42regularly exposed to poison, resulting in an acidic coating on their bodies [5].

43

44Although prophylactic sanitation of the nest and brood will limit microbial growth
 45[1,7], ants also use their poison defensively as a chemical weapon against predators
 46and other ants, and die from it themselves when exposed to high dosages, so it has the
 47potential to cause self-harm and collateral damage within the colony. Whilst the adults
 48and larvae have relatively robust cuticles, and the eggs a chorion, the pupae may be
 49particularly susceptible to the poison because their cuticles become fragile and thin
 50during metamorphosis. We therefore speculated that the pupae should be protected so
 51that the ants can use their poison without causing harm to the colony. Specifically, we
 52tested whether the silk cocoon, spun by larvae before pupation, functions as a
 53protective barrier, similar to humans utilising protective equipment to avoid harm
 54when using disinfectants.

55

56We created “nude” pupae by removing their cocoons and keeping both nude and
 57cocooned pupae with or without tending workers. Whilst the survival of cocooned and
 58nude pupae was unaffected when kept alone, in the presence of ants, more than twice
 59as many nude pupae died than cocooned pupae (Figure 1B; interaction_{w/wo cocoon*w/wo}
 60workers: LR- $\chi^2 = 5.29$, $df = 1$, $P = 0.02$; post hoc results in figure), despite the fact that
 61workers treated both pupal types equally (Figure S1) and they receive similar amounts
 62of acid [6]. Hence, the cocoon shields pupae from the activity of the tending ants.

63

64To test whether poison spraying by the ants causes nude pupa mortality, we created
 65functional “knockout” ants that were unable to spray poison, by gluing the opening of

66their poison glands shut. Whilst nude pupae kept with ants possessing functional
67poison glands showed high mortality, nude pupa survival was rescued in the knockout
68treatment where workers could not apply poison (Figure 1B; $\text{LR-}\chi^2 = 11.02$, $\text{df} = 1$, P
69= 0.0001). Poison spraying by tending workers therefore induces nude pupae
70mortality.

71

72We speculated that the acids in the poison damages the pupal cuticle and/or has other
73cytotoxic effects. Indeed, we confirmed that it is the antimicrobially active, acidic
74fraction of the poison killing the pupae, by applying synthetic ant poison consisting
75only of formic and acetic acid [5] onto pupae. We found that the poison causes nude
76pupae mortality in a dose-dependent manner, with some pupae dying at low dosages
77(Figure S2 and statistics therein). The cocoon, however, protected pupae against the
78acids, even at high dosages (Figure S2), with cocooned pupae surviving as well as
79water-treated controls (Figure 1D; interaction $\text{w/wo cocoon} * \text{w/wo poison}$: $\text{LR-}\chi^2 = 10.23$, $\text{df} = 1$,
80 $P = 0.001$, post hoc results in figure).

81

82Combined, our results reveal that ants prophylactically disinfect their colony with a
83poison lethal to the pupae, but that the latter are protected from its use by their
84cocoon. This protective effect is likely due to the hydrophobicity of the silk, which
85prevents the poison from entering the cocoon [6]. Hence, although constructing a
86cocoon is costly – delaying maturation by several days [8] – it ensures pupae can
87survive nest disinfection. Thus far, the function of ant cocoons and their presence in
88some but not all species has remained largely untested, but cocoon spinning is present
89in many ant subfamilies where acidic poison has evolved [9] and is used as a
90disinfectant [5,7]. We therefore hypothesise that poison spraying may be maintaining
91the need for cocoon spinning in ants that use harmful chemicals to disinfect their
92colonies, and, in turn, these species may have evolved a more potent poison by having
93cocoon-spinning brood, compared to species that do not. Curiously, the fungus-
94growing ants cover their pupae in the mycelia of symbiotic fungi [9]. Since these ants
95use caustic antimicrobials extensively in their nests to protect their fungal symbionts,
96but silk spinning is absent, such “makeshift cocoons” may also protect pupae from
97chemical disinfection. However, collateral damage may also be prevented through
98other mechanisms, such as selective poison use, and further comparative work is
99required to fully understand the factors selecting for cocoon spinning in ants.

100

101In conclusion, garden ants, by possessing protected brood, appear to have overcome
102the potential harm disinfectants can cause without compromising on their potency.
103Preventing self-harm whilst guarding against pathogens therefore appears to be a key
104tenet of disease defence, both at the level of individual immunity to minimise damage
105to healthy tissue (immunopathology) during an immune response [10], and social
106immunity, to protect the colony as a whole without harming its more fragile members.
107

108**Acknowledgements**

109We thank the *Social Immunity* group at IST Austria for help with ant collection and R.
110B. Rosengaus and an anonymous referee for their helpful comments on an earlier
111version on the manuscript. This study was funded by a European Research Council
112Starting Grant to S.C. (240371) under the European Commission's Seventh
113Framework Programme.

114

115**Author contributions**

116C.D.P. and S.C. designed the study and wrote the manuscript. C.D.P., S.M. and E.N.
117performed experiments and C.D.P. the data analysis.

118

119**Declaration of interests**

120The authors declare no competing interests.

121

122**Ethics**

123*L. neglectus* is an unprotected insect species and all ant collection and experiments
124comply with European laws and IST Austria ethical guidelines.

125

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156 **Figure title and legend**

157

158 **Figure 1. Cocoons protect pupae against the lethal side effects of colony**

159 **disinfection in ants.** (A) Garden ants spray poison in their colonies over time (paired
 160 data per nest displayed; inset shows nest floor example 2 d after moving in with pink
 161 areas of pH sensitive paper indicating poison use). (B) Nude pupae have lower
 162 survival in the presence of ants than cocooned pupae (despite the absence of any
 163 behavioural differences by the workers; see Figure S1). (C) Keeping pupae with
 164 acidopore-blocked ants improved nude pupae survival compared to pupae kept with
 165 ants able to spray poison. (D) Spraying pupae with synthetic ant poison (formic and
 166 acetic acid) decreased the survival of nude pupae only, whereas the water-sprayed
 167 controls were unaffected (which was also true for lower poison dosages; see Figure

168S2). Error bars show \pm 95% confidence intervals and letters denote significantly
169different groups ($P < 0.05$; Tukey post hoc test in (B) and (D), GLM result in (C)).
170

171**Supplementary Information**

172Supplementary Figures S1 and S2, Supplemental experimental procedures,
173supplemental references.
174

175**Deposited Data**

176All data supporting this work is available through Mendeley Data:
177Reserved DOI: doi:10.17632/pnkcjryfp9.1
178Link for review: [https://data.mendeley.com/datasets/pnkcjryfp9/draft?a=19f66abf-](https://data.mendeley.com/datasets/pnkcjryfp9/draft?a=19f66abf-179adfa-4655-8b60-e8364b5caff4)
179adfa-4655-8b60-e8364b5caff4