

# Ants defend aphids against lethal disease

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**Social insects defend their own colonies and some species also protect their mutualist partners. In mutualisms with aphids, ants typically feed on honeydew produced by aphids and, in turn guard and shelter aphid colonies from insect natural enemies. Here we report that *Formica podzolica* ants tending milkweed aphids, *Aphis asclepiadis*, protect aphid colonies from lethal fungal infections caused by an obligate aphid pathogen, *Pandora neoaphidis*. In field experiments, bodies of fungal-killed aphids were quickly removed from ant-tended aphid colonies. Ant workers were also able to detect infective conidia on the cuticle of living aphids and responded by either removing or grooming these aphids. Our results extend the long-standing view of ants as mutualists and protectors of aphids by demonstrating focused sanitizing and quarantining behaviour that may lead to reduced disease transmission in aphid colonies.**

**Keywords:** ant–aphid interactions; social insects; behaviour; mutualism; entomopathogenic fungi; common milkweed (*Asclepias syriaca*)

## 1. INTRODUCTION

Resource acquisition practices such as farming and animal husbandry are practised by social insects. Fungus farming by specific groups of ants has been intensively studied and shown to rely on ancient coevolved obligate mutualistic relationships (Mueller *et al.* 2005; Schultz & Brady 2008). Their success appears to have been associated with active sanitation practices, involving detection and removal of infectious microbes from their gardens (Currie & Stuart 2001) and domestication of antibiotic-producing filamentous bacteria (Poulsen *et al.* 2005; Currie *et al.* 2006).

Aphid husbandry by ants is generally considered to represent a less specialized association than that observed in fungus-farming ants. Interactions between ants and aphids range from mutualistic to antagonistic (Billick *et al.* 2007), and aphid tending by ants is usually facultative, which implies that ants maintain groups of phloem-feeding phytophagous insects without giving up their additional predatory and scavenging practices. Ants tending aphids feed on sugar-rich honeydew excreted by the sap-feeding

aphids and typically return benefits by guarding these herbivores from predators and parasitoids (Stadler & Dixon 2005, 2008).

Despite the abundant literature reporting beneficial effects of ant tending to protect aphids from insect enemies, no studies have investigated whether ants protect aphids they are tending from obligate fungal pathogens. One laboratory study has shown that ants remove aphid cadavers after infection by a non-epizootic facultative fungal entomopathogen, *Lecanicillium longisporum* (Bird *et al.* 2004). The obligate, aphid-specific fungal pathogen *Pandora neoaphidis* from the order Entomophthorales regularly causes epizootics in aphid colonies leading to local extinctions of colonies within a few days (Steinkraus 2006). Infection of aphids by *P. neoaphidis* is initiated when conidia land on a susceptible individual and penetrate the cuticle. A few days after infection, hosts die and new conidia produced on the cadaver's surface are actively ejected to infect healthy aphids (Pell *et al.* 2001). Thus, *P. neoaphidis* is externally present on affected aphids, both when conidia are first deposited on the cuticle as well as after killing the aphid, when the fungus grows out through the cuticle to discharge conidia. In this study we tested the efficiency with which *Formica podzolica* ants can recognize and remove milkweed aphids, *Aphis asclepiadis*, that are covered with conidia or have been killed by *P. neoaphidis*.

## 2. MATERIAL AND METHODS

We introduced experimental aphids into naturally established colonies of the milkweed aphid feeding on *Asclepias syriaca* in Ithaca, NY, USA. All aphid colonies were actively tended by workers of *F. podzolica*, an ant species that is a mutualist with some aphids (including *A. asclepiadis*), but also preys on insects, including their mutualist aphids (Mooney & Tillberg 2005). *Aphis asclepiadis* were inoculated with *P. neoaphidis* conidia following standard procedures (see electronic supplementary material).

### (a) Ant response to fungal-killed aphids

Each group of experimental aphids included three treatments: (i) fungal-killed *A. asclepiadis* cadavers producing conidia of *P. neoaphidis*, (ii) cadavers of *A. asclepiadis* killed by freezing, or (iii) living uninfected *A. asclepiadis* individuals. From each group of treatments, experimental aphids were introduced to aphid colonies in a random order, with at least 30 min between introductions. Ant behaviour, including removal of aphids or aphid cadavers, was recorded until the experimental aphid was removed from the colony or for 5 min if the aphid was not removed. Each set of three experimental treatments was repeated with 13 colonies two to six times on separate days ( $n = 41$  for each treatment).

### (b) Ant response to living aphids externally contaminated with conidia

Each pair of experimental aphids included: (i) living *A. asclepiadis* with high concentrations of *P. neoaphidis* conidia on the cuticle, and (ii) living non-inoculated *A. asclepiadis*. Each pair of experimental treatments was introduced to 26 colonies of aphids one to four times on separate days ( $n = 36$  for *P. neoaphidis* inoculated aphids and  $n = 39$  for non-inoculated aphids). After placement of an experimental aphid in a colony, the behaviour of ants entering the aphid colony was recorded. The frequency of tending events and the time that experimental aphids were tended (=moving the antennae over the aphid body), as well as the ant behaviour following directly after each tending event was recorded. Ant behaviour was recorded until the experimental aphid was removed from the colony or for 5 min if the aphid was not removed (for further details see electronic supplementary material).

## 3. RESULTS

### (a) Ant response to fungal-killed aphids

The overall removal behaviour of the ants varied depending on the type of experimental aphid placed in

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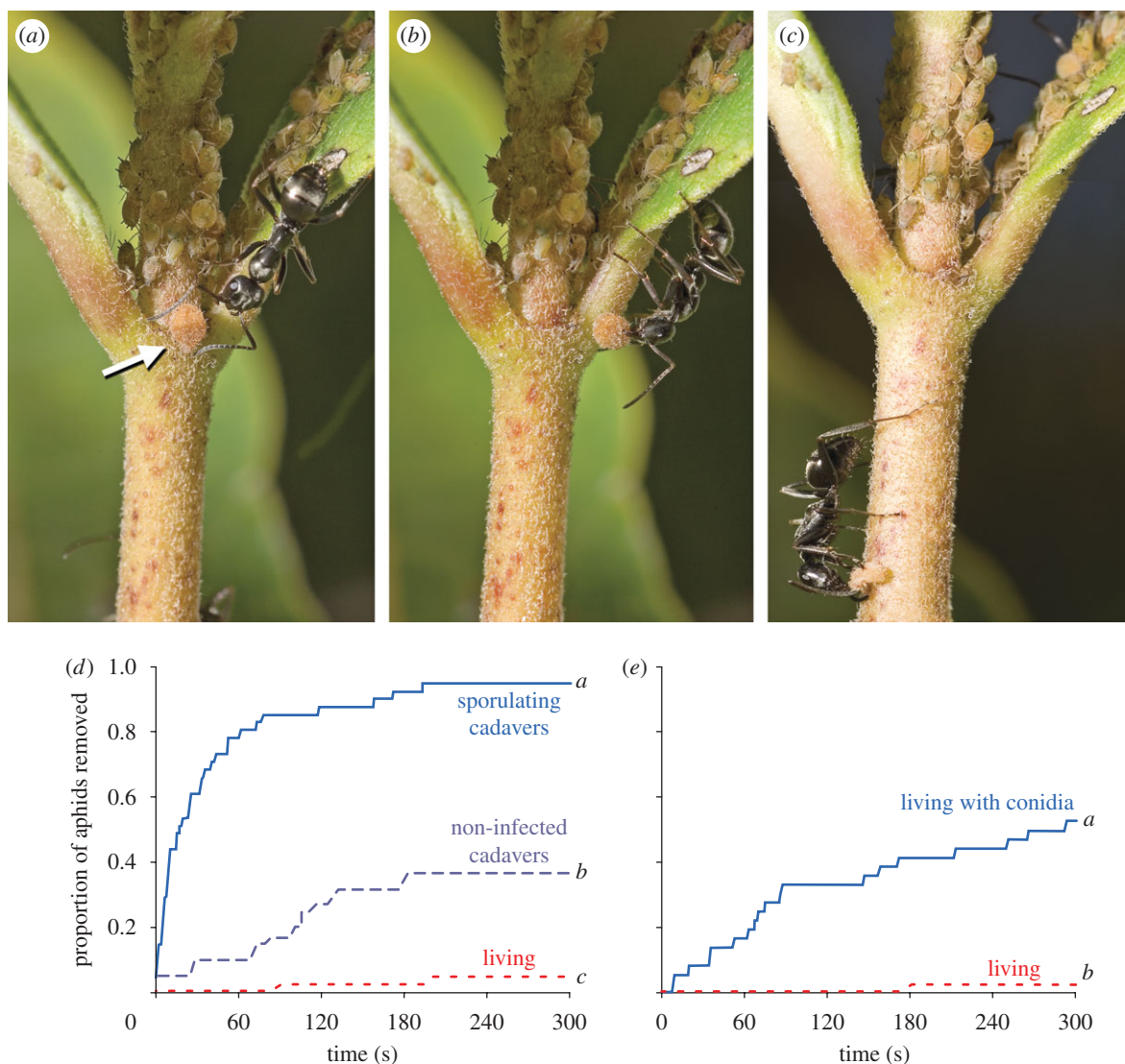


Figure 1. Removal of *Pandora neoaphidis*-killed *Aphis asclepiadis* cadavers by *Formica podzolica* ants. (a)–(c) Time sequence of an ant worker picking up a cadaver and carrying it down the stem of a milkweed plant (*Asclepias syriaca*). Sporulating cadaver indicated by arrow in photo (a) (photo: K. Loeffler). (d,e) Proportion of experimental aphids removed as a function of time. Different letters indicate significantly different removal curves at  $p < 0.05$  in pair-wise comparisons using Kaplan–Meier survival analysis.

the aphid colony (Wald = 60.70, d.f. = 2,  $p < 0.0001$ ). Cadavers of fungal-killed aphids were removed more frequently than non-fungal cadavers (Wald = 38.99, d.f. = 1,  $p < 0.0001$ ) and living aphids (Wald = 29.61, d.f. = 1,  $p < 0.0001$ ) (figure 1a–d). Thus, 95 per cent of the fungal-killed aphids were removed within 5 min of their introduction, whereas only 37 and 5 per cent of non-fungal cadavers and living aphids were removed, respectively. The proportion of aphids removed was independent of the number of aphids in the colony (Wald = 0.07, d.f. = 1,  $p = 0.7874$ ) but experimental aphids were removed more frequently when there were more ants tending the aphid colony (Wald = 5.89, d.f. = 1,  $p = 0.0152$ ).

Among the cadavers of fungal-killed aphids, 39 per cent were flung from leaves, 34 per cent were carried down the stem and 22 per cent were placed more than 1 cm away from the aphid colony. Thus, 73 per cent ended on the ground compared with 20 per cent of non-fungal cadavers ( $\chi^2 = 75.09$ , d.f. = 2,  $p < 0.0001$ ). None of the living aphids ended on

the ground. In the few instances when living aphids were moved, they were carried elsewhere on the plant.

The removal distribution of the three groups of experimental aphids differed significantly (Breslow = 99.17, d.f. = 4,  $p < 0.0001$ ); cadavers of fungal-killed aphids were removed faster than non-fungal cadavers and living aphids. The fungal-killed aphids had a hazard ratio of removal 7.7 times higher than non-fungal cadavers (Wald = 38.99, d.f. = 1,  $p < 0.0001$ ) and 91.9 times higher than the living experimental aphids (Wald = 29.61, d.f. = 1,  $p < 0.0001$ ).

The proportion of ants picking up an experimental aphid versus only touching it differed significantly among fungal-killed aphids ( $0.80 \pm 0.05$ ), non-diseased cadavers ( $0.19 \pm 0.05$ ) and living aphids ( $0.03 \pm 0.02$ ) (pair-wise comparisons: LS means  $\chi^2 \geq 5.84$ , d.f. = 1,  $p < 0.0157$ ). Ants that picked up a sporulating cadaver but failed to remove it from the aphid colony often left the aphid colony and spent time grooming themselves afterwards, whereas this grooming activity was rare for ants encountering a non-diseased cadaver (C.N. 2005, unpublished data).

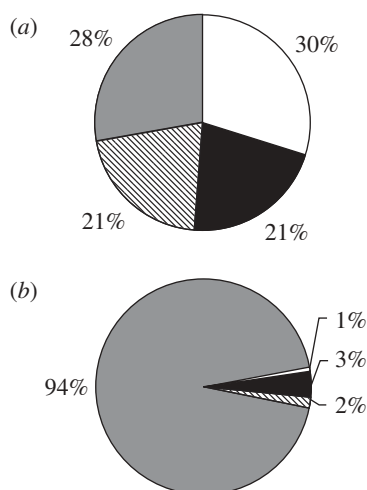


Figure 2. Percentage of different behavioural responses by *Formica podzolica* immediately after each aphid-tending event. (a) Living *Pandora neoaphidis*-inoculated *Aphis asclepiadis* placed in an aphid colony ( $n = 90$  aphid-tending events); or (b) non-inoculated *A. asclepiadis* placed in an aphid colony ( $n = 120$  aphid-tending events). No shading denotes self-grooming; black shading, aphid grooming; grey shading denotes no reaction; hatching denotes aphid removal.

#### (b) Ant response to living aphids contaminated with conidia

The removal distribution of conidia-contaminated aphids and uncontaminated aphids differed significantly (Breslow = 33.46, d.f. = 3,  $p < 0.0003$ ) with more conidia-contaminated aphids being removed at a faster rate than uncontaminated aphids. Living aphids contaminated with conidia had a hazard ratio of removal 17.7 times higher than uncontaminated aphids (Wald = 14.98, d.f. = 1,  $p < 0.0001$ ; figure 1e). Removal was independent of the number of aphids in the colony (Wald = 1.90, d.f. = 1,  $p = 0.1676$ ) and there was a tendency towards aphids being removed faster when there were more ants tending the colony (Wald = 3.41, d.f. = 1,  $p = 0.0649$ ).

Neither the frequency with which an experimental aphid was tended ( $F_{1,74} = 2.21$ ,  $p = 0.1416$ ), the total time the ant spent tending the aphid ( $F_{1,74} = 0.26$ ,  $p = 0.6130$ ), nor the time spent on each aphid-tending event ( $F_{1,59} = 2.10$ ,  $p = 0.1530$ ) were significantly affected by whether or not *P. neoaphidis* conidia occurred on the cuticle of the living experimental aphid. The ant behaviour immediately after tending an experimental aphid differed depending on the type of experimental aphid encountered (figure 2) ( $\chi^2 = 108.83$ , d.f. = 1,  $p < 0.0001$ ). Ants more frequently performed self-grooming after tending a conidia-contaminated aphid versus tending an uncontaminated aphid. Self-grooming was performed by pulling the antennae and forelegs through the mouthparts. Aphid-grooming was more frequent after tending conidia-contaminated aphids compared with non-contaminated aphids. In 21 per cent of instances when conidia-contaminated experimental aphids were picked up by the ant using its mandibles, the aphid was held between the forelegs and groomed, mainly using the mouthparts, all the while being rotated. Altogether, some means of sanitary action (removal,

self-grooming or aphid-grooming) was performed after 72 per cent of aphid-tending events for conidia-contaminated aphids as opposed to only 6 per cent for uncontaminated aphids ( $\chi^2 = 25.15$ , d.f. = 1,  $p < 0.0001$ ).

#### 4. DISCUSSION

Previous studies of ants tending aphids have reported protection of aphids from insect predators and parasitoids (Stadler & Dixon 2005) and removal of dead aphids from colonies (Flatt & Weisser 2000). This is the first report documenting that ants physically remove obligate aphid fungal pathogens from their aphid husbandry system. We report that defences against disease among aphid mutualists involved rapid removal of sporulating corpses, extensive aphid grooming to remove infective conidia and self-grooming after ant workers had been in contact with infective conidia on the cuticles of living aphids.

The physical removal of aphid pathogens and the sanitary behaviour of facultatively tending ants resemble the behaviour reported for obligate fungus-growing ants protecting their fungus gardens from unwanted microbes. The ants that we studied groomed fungal-contaminated aphids more frequently than uncontaminated aphids. Similarly, fungus-growing ants groom their fungus gardens more frequently when the fungus gardens are contaminated by alien fungi (Currie & Stuart 2001).

Under laboratory conditions, ants remove cadavers of the rosy apple aphid, *Dysaphis plantaginea*, sporulating with *L. longisporum* (Bird *et al.* 2004), a fungus that does not cause epizootics in nature. Our results document that advanced defences against disease also operate for obligate aphid pathogens under field conditions. *Formica podzolica* ants removed sporulating aphid cadavers faster than the *L. niger* ants studied by Bird *et al.* (2004). This may be related to *P. neoaphidis* being able to cause devastating epizootics, whereas the pathogen studied by Bird *et al.* (2004) is facultative and less virulent. These results match with those of fungus-growing ants, which react more strongly to infections by aggressive, specialized pathogens compared with general fungus garden weeds (Currie & Stuart 2001).

Hygienic behaviour practiced by ants towards nest-mates benefits ant colonies (e.g. Ugelvig & Cremer 2007). We found that facultatively aphid-tending ants practiced hygienic behaviour towards the aphids they tend, including rapid removal of conidia-producing aphid cadavers and conidia-contaminated living aphids, as well as extensive aphid grooming to remove infective conidia and self-grooming after ant workers had been in contact with conidia. We hypothesize that both ant and aphid mutualists benefit from this hygienic behaviour and that *F. podzolica* ants have been under selection to recognize diseased aphids and express this array of disease-management behaviours. Although colonies of these ants can survive without aphids, we hypothesize that aphid husbandry provides benefits such that multi-layered defences against epidemic disease of aphid livestock have evolved. This also suggests the possibility that this

fungal pathogen could act to reinforce ant–aphid mutualism, as has been suggested in the case of parasitism stabilizing the cooperative relationship between fungus-gardening ants and the fungus they garden (Little & Currie 2009).

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