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TITLE:

A Visual Guide for Studying Behavioral Defenses to Pathogen Attacks in Leaf-Cutting Ants

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SHORT ABSTRACT:

We present a visual guide to disease defense behaviors in leaf-cutting ants, with individual clips and accompanying definitions, illustrated in an experimental infection scenario. Our main aim is to help other researchers recognize key defensive behaviors and to provide a common understanding for future research in this field.

LONG ABSTRACT:

The complex lifestyle, evolutionary history of advanced cooperation, and disease defenses of leaf-cutting ants are well studied. Although numerous studies have described the behaviors connected with disease defense, and the associated use of chemicals and antimicrobials, no common visual reference has been made. The main aim of this study was to record short clips of behaviors involved in disease defense, both prophylactically and directly targeted towards an antagonist of the colony following infection. To do so we used an infection experiment, with sub-colonies of the leaf-cutting ant species *Acromyrmex echinatior*, and the most significant known pathogenic threat to the ants' fungal crop (*Leucoagaricus gongylophorus),* a specialized pathogenic fungus in the genus *Escovopsis*. We filmed and compared infected and uninfected colonies, at both early and more advanced stages of infection. We quantified key defensive behaviors across treatments and show that the behavioral response to pathogen attack likely varies between different castes of worker ant, and between early and late detection of a threat. Based on these recordings we have made a library of behavioral clips, accompanied by definitions of the main individual defensive behaviors. We anticipate that such a guide can provide a common frame of reference for other researchers working in this field, to recognize and study

these behaviors, and also provide greater scope for comparing different studies to ultimately help better understand the role these behaviors play in disease defense.

INTRODUCTION:

Leaf-cutting ants are advanced social insects, forming some of the most complex colonies on earth. They are a derived branch of the fungus-growing ants (tribe Attini) consisting of the two genera *Acromyrmex* and *Atta*[1]. They cultivate the fungal crop species *Leucoagaricus gongylophorus* (Basidiomycota: Agaricales), which they rely on as their main food source[2,3]. The ants supply this fungus with fresh leaf material for its growth, and the fungus in return produces nutrient-rich swollen hyphal tips (gongylidia) that are consumed by the ants and their brood. Colonies are built underground, the fungal crop is maintained in external gardens[4,5], and the ant farmers protect the monoculture from potential pathogens[6-12]. Colonies divide labor between workers of different size (caste) and age[13-15], which extends to the defense of ants and crop from pathogens.

We might expect that leaf-cutting ant colonies should be vulnerable to disease. Group living is expected to facilitate spread of diseases between related workers due to frequent interactions and, thus, easier transmission[16]. The ants are susceptible to entomopathogenic fungal parasites, such as *Metarhizium* species and *Beauveria bassiana*[6]. These parasites are generalists and are often present in the soil close to the nests[7,8]. Farming of the fungus crop as a monoculture[4,5] makes it likely to also be susceptible to disease[17,18]. It can be infected by generalist fungal parasites (including *Aspergillus niger* and *Trichoderma* species[3]); however, the most significant threat is a specialist necrotrophic fungus in the genus *Escovopsis* (Ascomycota: Hypocreales)[11]. Through secretion of mycolytic enzymes and other compounds, *Escovopsis* kills and obtains nutrients from the fungus crop[12], with potentially fatal consequences for the antcolonies[11,19].

To combat disease threats, the ants have remarkable defenses at both individual and colony level, combining biological control, behavioral and chemical defenses to act prophylactically and, when necessary, in response to infection. Collectively, these defenses prevent or reduce the impact of infections from both generalist pathogens and specialists such as *Escovopsis*. Broadly they involve avoiding the contraction of parasites in the environment[20], preventing parasites from entering the nest, and limiting the spread of infection within nests. The first lines of defense include chemicals from glandular secretions[3,21-27] to disinfect plant substrates, through workers licking them prior to incorporation in the fungus garden, and ants carrying out both self- and allogrooming. When grooming themselves, especially upon entering the nest, workers may also apply acidic fecal secretions to their body[27]. These prophylactic defenses are demonstrably important to avoid infection by pathogenic threats[6-12].

If initial defenses fail and a pathogen such as *Escovopsis* succeeds in entering the nest and the fungus garden, and if infection is detected at an early stage, the ants use fungus grooming to remove spores[25,28]. The ants may apply secretions from the metapleural glands or transfer the spores to the infrabuccal pockets (an oral cavity), where they are mixed with a chemical cocktail containing metapleural and labial gland secretions[26]. There are more than 20 known compounds in these glands, including γ-keto-, carboxylic- and indoleactic acids[3]. These are actively applied[25],

have antibiotic, fungistatic and fungicidal properties[29], and can inhibit *Escovopsis* spore germination[30]. Spores stored in the infrabuccal pocket are later expelled outside the colony[31,32]. Most of this fungus grooming following early-stage detection is carried out by minor workers[28,33]. However, if a pathogen manages to avoid detection and spreads further within the fungus garden, both minor and major workers weed infected parts of the fungus[28], and this removed material is deposited outside the nest[31]. In addition, species in the leaf-cutting genus *Acromyrmex* use biological control in the form of antibiotics produced by symbiotic Actinobacteria[34-36],— maintained on the ant cuticle[37] of predominantly young major workers[34,38-40] — to produce compounds that prevent mycelial growth of *Escovopsis*[34,38,41]. This antibiotic production may in turn be impaired by *Escovopsis*-produced compounds during an infection[19].

[PLACE FIGURE 1 HERE]

Leaf-cutting ant defenses thus constitute an integrated assembly of behavioral and chemical mechanisms that collectively provide these ants with very efficient protection from disease[42]. Understanding these defenses is of broad interest, and they have been extensively researched[16,20,42-44]. However, a visual compilation of the defensive behaviors that would help unambiguous definition and description of them for systematic use by researchers is, to our knowledge, not available. Although the terminology used to describe ant behavior is relatively standardized, there is therefore no certainty that the same behavioral acts are named consistently in different studies. Here, our main goal is to remedy this by providing clarity and standardization through a compilation of video recordings of individual prophylactic and defensive behaviors accompanied by associated definitions. We filmed these clips during a behavioral experiment, in which we observed and quantified behaviors in the context of experimental *Escovopsis* infections of *Acromyrmex echinatior* sub-colonies, the results of which we also present here as an illustrative example of how this compilation can be used for behavioral studies.

PROTOCOL:

1. ***Escovopsis* isolation**

1.1. Isolate *Escovopsis* strains from *Acromyrmex echinatior* field colonies, by growing spores removed from the colony waste dump or fungus garden on potato dextrose agar (PDA, 39 g/L) plates. Incubate at ca. 23°C for approximately two weeks.

Note: One strain was used in the current study.

1.2. Use a sterile needle to pick out mature spores from these first plates. Pick enough spores to cover the tip of the needle.

1.3. Inoculate spores on new plates under sterile conditions and incubate at ~ 23 °C for approximately two weeks.

1.4. When hyphae have covered the entire plate and grown into mature brown spores, repeat step 1.3 but this time with spores from the new PDA plate.

1.5. Repeat the process until a clean stock of *Escovopsis* for each strain is acquired (no visible contaminants growing on or below the plate surface).

2. **Experimental set-up**

2.1. Use three *A. echinatior* colonies.

Note: Here the colonies Ae160b, Ae322 and Ae263 were used.

2.2. From each colony, make 12 sub-colonies. Make six sub-colonies for observation after 0 h and six sub-colonies for observation after 72 h. This gives a total of 36 sub-colonies; mark half of the sub-colonies from each 'parent' colony as control, and the other half as '*Escovopsis* treatment'.

Note: While in full colonies the presence of a queen affects worker behavior, we expect (although this cannot be guaranteed), that queen-less sub-colonies are likely to behave as queenright colonies for the short period of time that the experiment runs.

2.3. For each sub-colony, use a square box of length: ~ 3.15 in (8 cm), width: ~ 2.17 in (5.5 cm) and depth ~ 1.77 in (4.5 cm).

Note: The important point is to provide enough space for ants outside the fungus fragment to forage and dump waste, but at the same time to make the box small enough that filming and behavior recognition is feasible.

2.4. For each sub-colony, approximately 4 h prior to ant introduction, add a tea-spoon sized (approximately 2.2 cm^3 and 1.2 g) piece of the central part of the fungus garden (*L. gongylophorus*) from the original colony, a couple of bramble leaves, and a piece of cotton wool soaked in water.

Note: The cotton wool provides humidity. It should not be dripping with water, and should not touch the fungus garden.

2.5. For sub-colonies treated with *Escovopsis*, use an inoculation loop and fill the opening so it is just covered by *Escovopsis* spores. Inoculate the spores by gently tapping a small, localized part of the fungus garden ten to twenty times so that spores are not too clustered.

2.6. For sub-colonies used as controls, mimic the application of *Escovopsis* to the fungus garden with a sterile inoculation loop.

Note: Although this was not done in the present experiment, the inoculation of a sterile powder (like graphite or talcum powder) can be done at this stage to differentiate between an infection with a pathogen and an inert agent.

2.7. For **72 h observations**, leave half of the sub-colonies (both controls and infected) for 72 h after introduction of *Escovopsis* before adding ants or video recording.

Note: Waiting 72 h in the absence of ants makes it likely that *Escovopsis* spores will germinate (unpublished *in vitro* data); although this also increases the chance of other infections (for example, from a fungus already present in the fungus garden), this time period is preferable for this treatment to represent the early stage of an established infection.

2.8. For **0 h observations**, directly after step 2.6 and approximately 30 min before recording, add two brood, four minor workers and four major workers simultaneously from the parent colony to each box.

2.8.1. Use two major workers from within the garden that are young with light pigment, with Actinobacteria covering most of the cuticle. Take the other two from outside the garden, with dark pigment and Actinobacteria only covering the laterocervical plates.

2.9. For **72 h observations**, repeat 2.8 and 2.8.1 at 30 min before recording, *i.e.*, 71.5 h after inoculation.

Note: Experimental sub-colonies are significantly smaller than established natural *Acromyrmex* colonies. This is necessary in order to accurately record behavior. While this may influence the frequency of some behaviors qualitatively, the composition of sub-colonies was chosen to reflect the mix of workers in natural colonies to more likely qualitatively reflect the behavioral interactions.

3. **Video recording and scoring behaviors**

3.1. Use a USB endoscope attached to a laptop (or equivalent) and provide sufficient light.

3.2. For each sub-colony, perform video recording for 4 h (starting at either 0 h or 72 h post-infection).

3.3. After recording the 36 sub-colonies, review the total of 144 h of footage and score all behaviors of interest for all individuals in each sub-colony.

Note: In the current experimental example, we had to exclude two sub-colonies (a control from colony Ae160b and a treatment sub-colony from colony Ae322) due to infection with fungi other than *Escovopsis*, reducing the total number of hours of observations to 136.

3.4. Each time a behavior is observed, record it as 1 occurrence.

Note: A behavior can be of short or long duration, but only score it as > 1 if it is interrupted by another behavior, or if the ant is passive for a period of time.

4. **Behaviors**

Note: Behavioral definitions were made using a combination of descriptions from previous studies[23,27,28,31,45] and personal observations. For a detailed illustration showcasing important morphological structures used in the protocol for recognition of behaviors, see **Figure 1**.

4.1. Self-grooming and antenna cleaning (Video 1)

4.1.1. Notice if the ant stops leg movement to initiate self-grooming. Check that the antennae are pulled through the antenna cleaners on the front legs (**Figure 1**), a clamp-like structure located on the tibia-tarsus joint consisting of a notch facing a spur with different sized bristles and combs[45,46].

4.1.2. After cleaning the antenna, observe that the ant will clean the legs and the antenna cleaners, by pulling the legs through the mouthparts, removing particles and potential pathogens with the glossa (**Figure 1**).

Note: Self-grooming can consist of cleaning the antenna and subsequently the antenna cleaners (**Figure 1**), but also using mouthparts to clean the legs. When an ant is cleaning its legs, it most often cleans all six legs in succession.

4.2. Fungus grooming (Video 2)

4.2.1 Notice if the ant stops leg movements at a fixed point on the fungus garden. Observe that the antennae are motionless and parallel pointed towards the fungus so that the angle between the scape and funiculus (**Figure 1**) is approximately 45°, and the tip of the antennae are almost touching each other, close to the tip of the mandibles (**Figure 1**).

4.2.2 Note that the upper (maxillae) and lower (labium) mouthparts are open, with the glossa (**Figure 1**) emerging to lick the fungus.

4.3. Allogrooming (Video 3)

4.3.1. Observe this behavior when one or more ants have approached another (recipient) ant or *vice versa*. During the behavior, the ants stop movement and stand closely together with physical contact. The grooming ant(s) may move slightly to cover a larger area of the recipient ant's body.

4.3.2. Observe that the antennae of the actor(s) can be motionless and pointed towards a specific point of the receiving ant or moving and lightly tapping the receiver. The angle between the scape and the funiculus (**Figure 1**) is approximately 45° depending on whether they are fixed on a

specific point or tapping. The tips of the actor's antennae are usually close to each other and the tip of the mandibles (**Figure 1**).

4.3.3. Note that the upper (maxillae) and lower (labium) mouthparts are open, with the glossa (**Figure 1**) emerging to lick the receiver ant.

4.4. Metapleural gland grooming (Video 4)

4.4.1. Observe when the ant stops movement to initiate metapleural gland (**Figure 1**) grooming. Note that the ant leans to one side to reach one of its front legs back to rub the opening (meatus) of the metapleural gland (for example, the right front leg).

Note: The other front leg is simultaneously (in this example the left front leg) licked by the glossa (**Figure 1**).

4.4.2. Check that the ant leans to the opposite side and switches legs and repeats the same motion with the opposite legs. The ant continues to move the front legs to the metapleural glands and subsequently to the glossa constantly switching between legs (**Figure 1**).

Note: In this example, the ant will now pass the left front leg to the metapleural gland and the right front leg to the glossa (**Figure 1**). After metapleural gland (**Figure 1**) grooming, the ant often initiates self-grooming (Step 4.1).

4.5. Spore weeding (Video 5)

4.5.1. Observe this behavior when the ant stops leg movements at a fixed point on the fungus garden. Observe that the antennae are motionless and parallel, pointed towards the fungus so that the angle between the scape and funiculus is approximately 45°, and the tips of the antennae are almost touching each other and the tip of the mandibles (**Figure 1**).

4.5.2. Check that the ant opens its mandibles (**Figure 1**) to grab visible *Escovopsis* spores and detaches them from the fungal crop by pulling them off. The ant carries the cluster of spores out of the nest, while the antennae are gently moving for orientation. The antennae may be cleaned through the antenna cleaners (**Figure 1**; see **Video 1**) while holding the cluster of spores. The ant drops the spores off in a waste pile.

Note: Recording of activity around the waste pile was not done in the current experiment but would be a suitable extension of the current protocol.

4.6. Fungus weeding (Video 6)

4.6.1. Observe this behavior when the ant stops leg movements at a fixed point of the fungus. The antennae are loosely pointed towards the part of the fungus the ant is attempting to remove, while slightly tapping the fungus piece.

4.6.2. Observe that the ant uses its mandibles to either cut through the fungal crop to detach a specified area, or to grab a part of the fungus piece with its mandibles (**Figure 1**). The ant will simultaneously rock from side to side on its legs, while it pulls off the fungus piece.

Note: Weeding can be done by multiple workers and by both minors and majors. If so, some ants do the fungus cutting, others carry out the rocking and pulling motion. The detached part of the fungus is carried outside the nest and dropped off in the waste pile. Recording of the waste deposit was not done in the current experiment but would be a suitable extension of the current protocol.

4.7. Fecal fluid grooming (Video 7)

4.7.1. Observe this behavior when the ant stops leg movements at a fixed point on the fungus garden. The ant bends its gaster (**Figure 1**) and head towards each other to apply a droplet of fecal fluid to the mouthparts.

4.7.2. Observe that the ant pulls the front legs through the mandibles (**Figure 1**), one at a time. Subsequently, the ant moves the antennae through the antenna-cleaners (**Figure 1**) located on the tibia-tarsus joint of the front legs.

4.8. Droplet regurgitation (Video 8)

4.8.1. Observe this behavior when the ant stops leg movements at a fixed point of the fungus. The antennae are motionless and parallel, pointed towards the fungus so that the angle between the scape and funiculus is approximately 45°, and the tips of the antennae almost touch each other and the tip of the mandibles (**Figure 1**).

4.8.2. Observe that the ant regurgitates a droplet of liquid on to the fungus, varying from being transparent to light yellow or even brown, from its mouthparts.

REPRESENTATIVE RESULTS:

The main objective of this study was the creation of short clips that illustrate behaviors associated with disease defense in leaf-cutting ants, to generate a catalogue to be used as a reference for future studies. In addition, the study uses an example of an experimental setup within which these behaviors were quantified to show how this catalogue may be used in behavioral studies, the representative results of which we summarize here.

Sub-colonies were set up for observation at early stage (0 h) and late stage (72 h) infection. Due to heavy infections with fungi other than *Escovopsis* after 72 h, two sub-colonies (one control for colony Ae160b and one treatment for colony Ae322) were excluded, so we focus the presentation of the results on the 0 h time point and put less emphasis on behaviors observed after 72 h. After filming all sub-colonies and scoring the defensive behaviors, we found differences in behavior patterns associated with time after infection and context, including the

different ways in which they were used by minor and major workers. In contrast, self-grooming was performed at all times in both control and infected sub-colonies. It was also common when ants were seeking, and attempting to remove, *Escovopsis* hyphae or spores. Because this behavior was so universally observed and frequent in all situations, we did not quantify it.

We scored all other behaviors described in the protocol and present calculated total and average frequencies for minor and major workers per sub-colony (**Table 1**). We found that in controls, minor workers groomed the garden crop more than major workers (**Figure 2a**), and anecdotally observed that they spent more time in the fungus garden. In colonies infected with *Escovopsis* there was a tendency for increased fungus grooming overall relative to control colonies, but this was not significant ($F_{1,23}$ = 2.80, p = 0.1077; **Table 2**; **Figure 2a**). There was a non-significant increase in fecal grooming with infection ($F_{1,23}$ = 0.60, p = 0.4455; **Table 1**) but no difference between minor and major workers (**Figure 2b**). We observed fecal grooming when workers entered the fungus, rather than when they were on or completely away from the fungus for extended periods of time.

[PLACE FIGURE 2 HERE]

Several behaviors were extremely rare. We only observed metapleural gland grooming four times, and all instances occurred after 72 h; once in the control group, and three times in colonies with *Escovopsis* infection (**Table 1**). Almost as infrequently, we found that ants would regurgitate a liquid droplet onto the fungus garden and a few times outside of the fungus (**Table 1**). In the 0 h groups, this happened once in a control sub-colony and twice in infected colonies — in all three instances the behavior was performed by a major worker. On one occasion a minor worker regurgitated a droplet in the corner of the box and once a major worker did the same on a bramble leaf, both in the *Escovopsis* treatment. In the 72 h colonies, droplet regurgitation on the fungus was never observed in the control colonies but happened seven times in infected colonies. Six of these were by major workers and three of these by a single individual that added droplets outside of the fungus garden.

Spore weeding (**Figure 3a**) and fungus weeding (**Figure 3b**) were both low in frequency. While there was no significant difference in the frequency of fungus weeding between infected colonies and controls, there was a tendency for weeding to increase with time since infection ($F_{1,23}$ = 2.91, p = 0.1014; **Table 2**). Our observations focused on *Escovopsis* spore weeding, which did not change significantly with time, but there was a tendency for a higher frequency in colonies infected with *Escovopsis* than in uninfected controls ($F_{1,23}$ = 3.27, P = 0.0838; **Table 2**). Fewer sub-colonies infected with *Escovopsis* had visible spores remaining after the observation period with an early-stage infection (when ants were introduced after 0 h of spore inoculation), with ants removing all spores in almost half of the sub-colonies (4 out of 9; **Figure 3c**). In later stage infections (where ants were introduced after 72 h of spore inoculation), the ants were not capable of removing spores completely in any of the sub-colonies. Taken together, these observations suggest a tendency for ants to shift from spore weeding to fungus weeding over the course of an infection.

[PLACE FIGURE 3 HERE]

FIGURE AND TABLE LEGENDS:

Figure 1: Ant morphological features. A schematic drawing of an ant showing the morphological structures mentioned in the protocol.

Figure 2: Frequency of grooming events. Mean number (± Standard Errors (SE); n = 9) of **(a)** fungus grooming and **(b)** fecal fluid grooming events within four hours after inoculation, comparing minor and major workers in controls and *Escovopsis* treatments.

Figure 3: Frequency of weeding events. Mean frequency (± Standard Errors (SE); n = 9) of **(a)** spore weeding (of *Escovopsis* spores) and **(b)** fungus weeding (*Escovopsis* or other fungi) during a 4 h observation period, comparing minors and majors from control and treatment groups, and **(c)** the number of sub-colonies with visible *Escovopsis* spores in treatment groups at the end of the observation period.

Table 1: The number of behaviors in four hours of observation at 0 and 72 h after *Escovopsis* inoculation. The mean total number of observations (with mean frequencies per individual in brackets), comparing minor and major workers from control and *Escovopsis* infection sub-colonies, respectively (n = 9).

Table 2: Statistical results of the mixed ANOVA tests on separate behaviors for which statistical analyses could be performed. Fixed effects were colony, sub-colony (nested within colony), treatment, and time.

Video 1: Self-grooming and antenna cleaning.

Video 2: Fungus grooming.

Video 3: Allogrooming.

Video 4: Metapleural gland grooming.

Video 5: Spore weeding.

Video 6: Fungus weeding.

Video 7: Fecal fluid grooming.

Video 8: Droplet regurgitation.

DISCUSSION:

The primary objective of this study was to observe and record characteristic leaf-cutting ant defensive behaviors in the presence of fungus-garden infection with *Escovopsis*, creating reference clips for use by the wider scientific community. It should be noted that these behaviors are not exclusive to defense of colonies from *Escovopsis*, but may also play a role in defense against other contaminants and infections[6-12], and in the defense of the ants themselves[42]. Our protocol provides a backdrop for wider research on defenses in fungus-growing ants. This is likely to be particularly useful: (i) for young researchers who are not familiar with these behaviors; (ii) to secure consistent definitions for and observations of behaviors; (iii) to facilitate comparisons across studies and ant species; (iv) because a number of these behaviors may occur so infrequently that even experienced researchers may never have observed them; (iv) because understanding and recognizing behaviors in controlled conditions in the laboratory help studies *in situ* where conditions are harder to control.

The results from our behavioral study are consistent with previous work which showed that minor workers fungus groom — crucial if an infection is detected early — more than major workers[25,28,32]. Here, major workers increased the amount of fungal grooming after *Escovopsis* infection (**Figure 2a**). This suggests that minor workers are the predominant fungus groomers, but that major workers may assist in preventing the spread of more established infections. The larger major workers can remove spores faster, while minor workers could be more suited to removing less accessible spores. We also found that workers successfully removed spores in around half of the infected sub-colonies (four out of nine) when they were introduced at the time of infection, and thus could detect the pathogen early (**Figure 3c**). Overall, this points to a series of behavioral responses where the ants first try to stop *Escovopsis* infection by removing spores (and doing so before an infection spreads), rather than removing parts of the fungus garden (**Figure 3a,b**). This changes over time if the infection progresses, when ants are more likely to remove parts of the fungus garden[28]. Although our sample sizes were too small to be conclusive, and we cannot rule out that simultaneous infections induced weeding behaviors, our data supports this trend, with fungus weeding predominantly being present at later stages of infection (**Figure 3a**). The generally low levels of fungus weeding might suggest either that the ants used other defenses (*e.g.*, chemicals) to inhibit further growth of *Escovopsis*, or that none of our experimental sub-colonies were too severely infected (making the more destructive defenses unnecessary).

Our findings suggest that self-grooming with fecal fluid is characteristic of ants entering the fungus garden, and used as a prophylactic measure, rather than being associated with an infection. Similar observations have been seen in foundress females that groom themselves and transfer fecal droplets with their mouth to their legs, when entering the nest or handling the crop[27]. An infection should in theory increase the activity of workers at the edge of the fungus garden, if the removed infected material is carried out and dropped in waste piles. Hence, fecal fluid grooming may also indirectly increase during infection to minimize disease spread. We would expect the opposite pattern for severe infections, with reduced movement at the edge of the fungus garden, as workers either abandon the fungus or adopt more extreme measures such as chemical defense.

While fecal fluids could serve as an important prophylactic chemical for an individual, allogrooming is used by nest-mates on other workers if they detect foreign particles or microbes. The substantial difference we observed between the frequency (**Table 1**) of fecal fluid grooming (n = 304) and of allogrooming (n = 48) might indicate a difference in pathogen detection. Ants are not able to easily detect pathogens on themselves with their antennae; allogrooming on the other hand is done by nest-mates, who can inspect the entire body of an ant and only choose to groom if necessary. Since *Escovopsis* is a parasite of the fungus garden rather than the ants, this might also explain the low amount of allogrooming.

We rarely observed metapleural gland grooming, and only at later stages of infection. Species of fungus-growing ants with abundant *Pseudonocardia* bacterial cover groom the metapleural glands less than species with less or no cover[25,47]. As *A. echinatior* has an abundance of the symbiont[47], this may explain the low gland grooming frequency. The metapleural gland secretion is also expensive to produce[30], and may be stored within the infrabuccal pockets for longer periods of time, meaning that grooming of the metapleural gland may be needed less often. During metapleural gland grooming, the ants simultaneously switch legs and lick the leg that had just groomed the gland; the spores are thereby transferred to the infrabuccal pockets, where gland secretions are critical for inhibiting *Escovopsis*' potential for subsequent germination[25]. Minor workers are more abundant inside the nest and have bigger metapleural glands per unit body mass[30], suggesting they are responsible for the majority of the metapleural gland secretions. This could also explain why, in our study, the highest frequency of fungus grooming was among minor workers.

We expected to observe behavior(s) indicating active use of the antibiotics from the bacterial symbiont *Pseudonocardia*, commonly observed on the cuticle of *Acromyrmex* workers and known to play a role in defense against *Escovopsis*[36,39,40]. The most likely explanation for not observing such behavior, is that the application of these antibiotics may be incorporated into other behaviors, such as self-grooming followed by fungus grooming and/or weeding, which may make it hard to observe as a distinct behavior.

We observed the unusual behavior of regurgitating liquid droplets on to the fungus garden. Regurgitation of food for nestmates has previously been described in leaf-cutting ants[22]. In our experiment, droplets differed in color from transparent to dark brown, suggesting they may be a food source for other ants and/or provide water. We only observed two occasions where other ants drank from the droplets, so we cannot determine if the droplets benefit other ants or serve to rehydrate the fungus when humidity is low. Most observations of this behavior were during *Escovopsis* infections, which might imply a defensive role, such as immune priming by regurgitation of anti-microbial peptides[16,48]. We cannot draw firm conclusions on this since this behavior was rare, but it would be an interesting line to investigate further, for example, by determining whether droplets have antimicrobial properties.

Given that observational studies of the complex defensive behaviors of leaf-cutter ants, including any comparison with and without fungus garden infection, would be extremely difficult to make in the field, experimental data can provide valuable insights into these behaviors under more

controlled conditions. While the observations made under laboratory conditions might differ from the behaviors found under natural conditions, tools such as our catalog of key defensive behaviors need to be developed, to improve both experimental and field studies in the future. The experimental approach may, however, partially explain why some behaviors were extremely rare (*e.g.,* allogrooming, metapleural gland grooming) in our demonstration of using these behavioral definitions. Future studies might therefore consider the limitations of this experimental setup, to find ways of making more naturalistic observations. Additional factors could also be integrated into the current protocol, such as distinguishing between Actinobacteria-carrying (younger) workers and older workers with less abundant cover, that may respond differently to the threat of an *Escovopsis* infection. There are trade-offs between making observations more accurate (for example by scoring focal individuals), or having larger sub-colony size (greater number of workers), and the amount of time or number of sub-colonies or individuals that can be filmed at a given point in time. Nevertheless, while the set-up could be extended for larger behavioral studies with a focus on addressing a behavioral goal, in this case we focused on successfully showcasing a method for recording and defining specific defensive behaviors.

We documented behaviors that contribute to defense in leaf-cutting ants, and more significantly, have systematically identified, described, and captured defensive behaviors on film. Our representative results reinforce other research in this field suggesting why it is hard for a pathogen to successfully infect fungus-farming ant colonies, when facing an extensive set of defensive behaviors and associated application of antimicrobial compounds. Our main goal was to provide a new tool for future work in this field, and we hope that the behavioral catalog will prove valuable for securing consensus and streamlined definitions, observations, and interpretations of behaviors, to serve as an important resource for future research.

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The authors have nothing to disclose.

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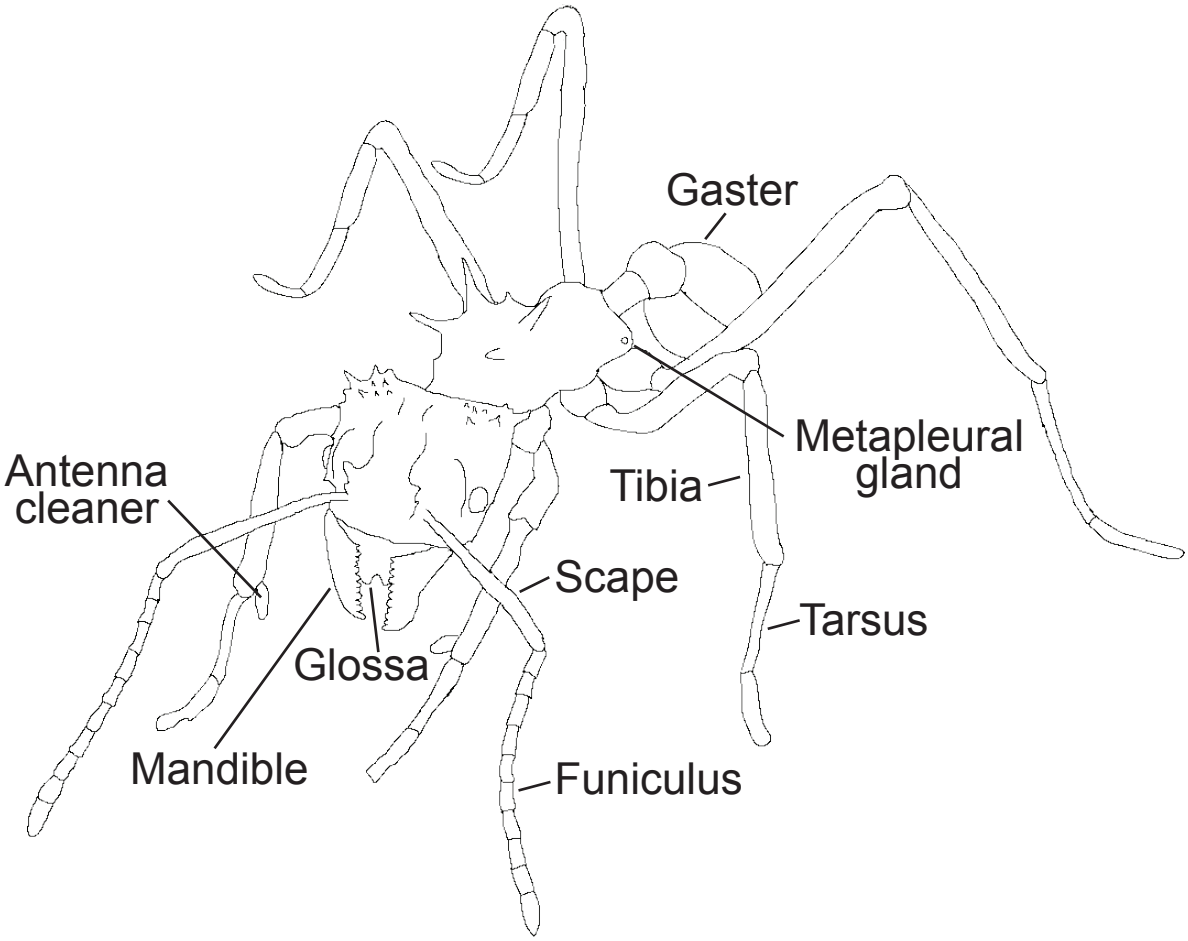

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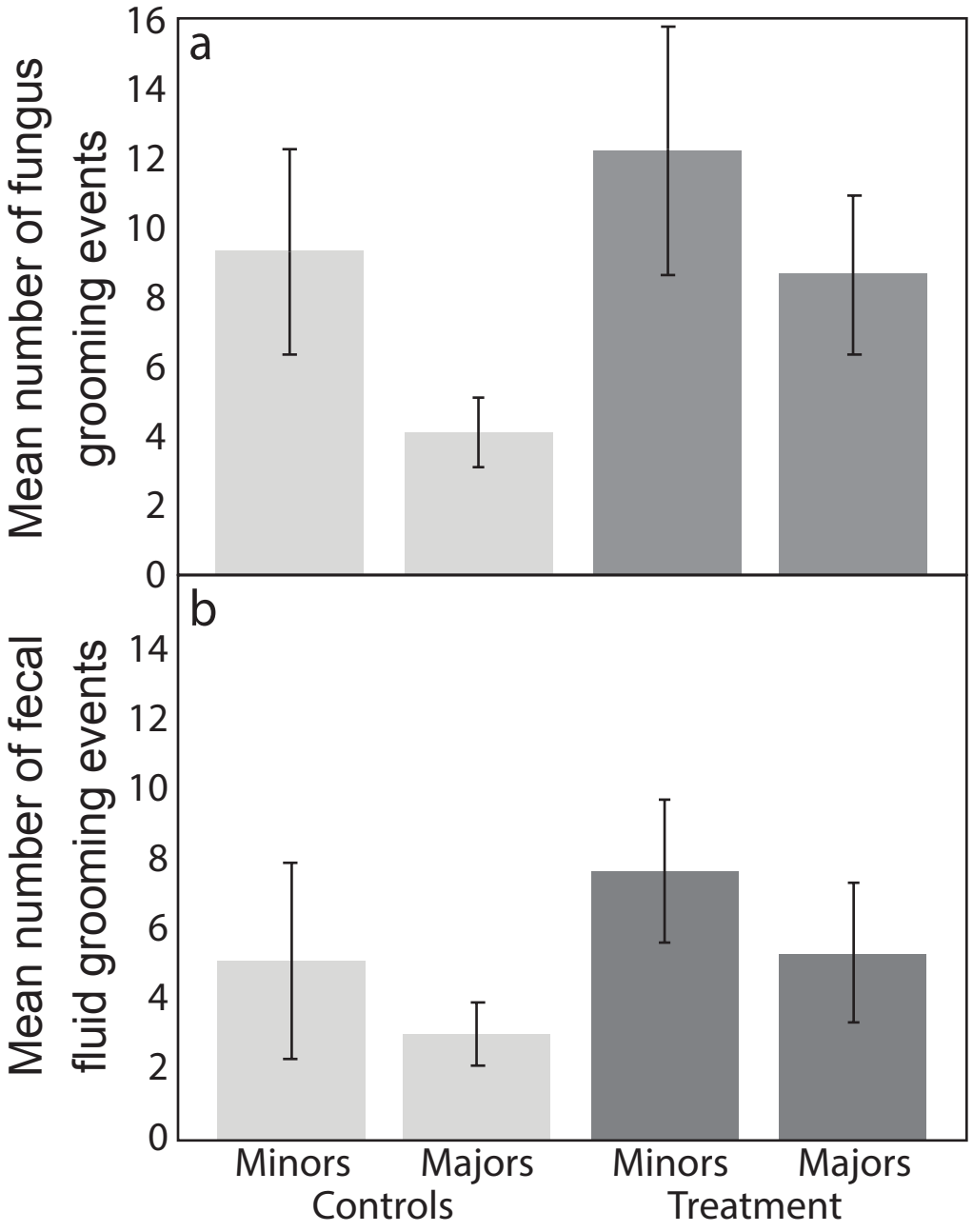

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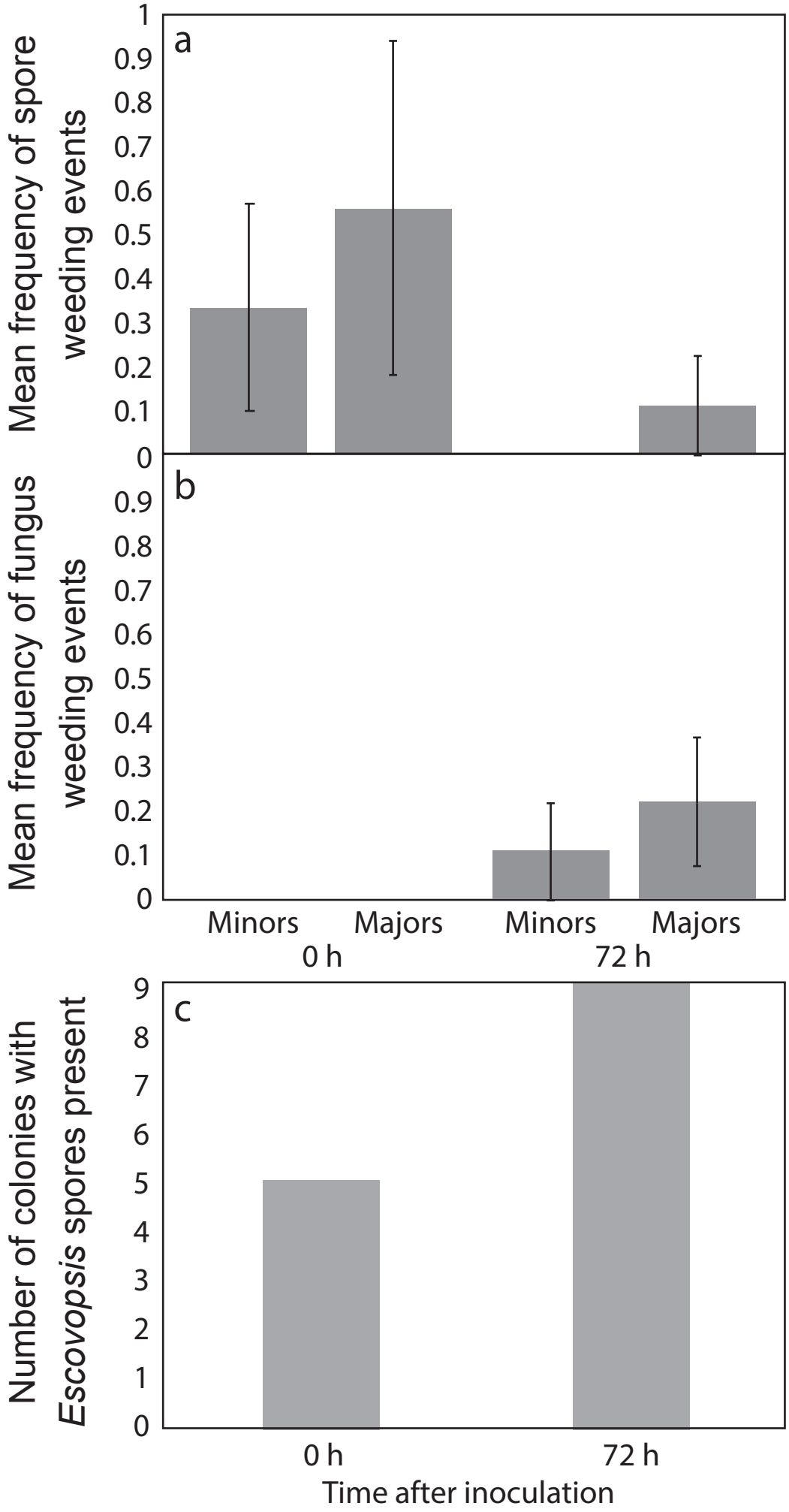

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Video 2 Fungus grooming Clip2

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Video 3 Allogrooming Clip3

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Video 4 Metapleural gland grooming Clip4

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Video 5 Spore weeding Clip5

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Video 6 Fungus weeding Clip6

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Video 7 Fecal fluid grooming Clip7

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Video 8 Droplet regurgitation Clip8

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Table 1: The number of behaviors in four hours of observation at 0 and 72h after *Escovop*
mean frequencies per individual in brackets), comparing minor and major workers from

	Minor workers - control	**Minor workers - treatment**
Fungus grooming	84 (9.333)	131 (14.56)
Allo-grooming	6 (0.6667)	14 (1.556)
Spore weeding	0	3
Fungus weeding	0	0
Fecal droplet grooming	46 (5.111)	69 (7.667)
Droplet application	0	0
Metapleural gland grooming	0	0
Fungus grooming	10 (1.429)	45 (5.000)
Allo-grooming	2 (0.2857)	10 (1.111)
Spore weeding	0	0
Fungus weeding	0	1 (0.1110)
Fecal droplet grooming	19 (2.714)	38 (4.222)
Droplet application	0	1 (0.1110)
Metapleural gland grooming	0	2 (0.2220)

***ɔsis* inoculation.** The mean total number of observations (with control and *Escovopsis* infection sub-colonies, respectively.

Major workers - control	Major workers - treatment
0h	
37 (4.111)	66 (7.333)
5 (0.5556)	6 (0.6667)
0	5 (0.5556)
0	0
27 (3.000)	48 (5.333)
1 (0.1111)	2 (0.2220)
0	0
72h	
24 (3.429)	38 (4.222)
1 (0.1110)	4 (0.4440)
0	1 (0.1110)
0	2 (0.2220)
30 (4.286)	30 (3.333)
0	6 (0.6667)
1 (0.1429)	1 (0.1110)

Table 2: Statistical results of the mixed ANOVAs on separate behaviors for which statistical analyses could be

									Typ
	Fungus grooming				Allogrooming				
Effects	**Num DF**	**Den DF**	**F value**	**Pr > F**	**Num DF**	**Den DF**	**F value**	**Pr > F**	**Num DF**
Colony	2	23	0.77	0.4733	2	23	0.52	0.5989	2
Sub-colony (colony)	6	23	0.93	0.4892	6	23	0.51	0.7978	6
Treatment	1	23	2.8	0.1077	1	23	1.85	0.1875	1
Time	1	23	6.53	0.0177	1	23	0.88	0.3574	1

performed. Fixed effects were colony, sub-colony (nested within colony), treatment, and time.

e 3 tests of fixed effects

Fecal grooming			Spore weeding				Fungus weeding			
Den DF	F value	Pr > F	Num DF	Den DF	F value	Pr > F	Num DF	Den DF	F value	Pr > F
23	0.54	0.5903	2	23	0.51	0.6052	2	23	1.17	0.3272
23	0.63	0.7067	6	23	1.67	0.1742	6	23	1.53	0.2127
23	0.6	0.4455	1	23	3.27	0.0838	1	23	1	0.3275
23	0.97	0.3361	1	23	0.53	0.4742	1	23	2.91	0.1014

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number
Plastic boxes	N/A	N/A
Petri dishes	Sarstedt	82.1472
Inoculation loops	Labsolute	7696431
Cameras	DBPower	NTC50HD_8.5mm
Holders for the cameras	N/A	N/A
Laptop	HP	N/A
Program used on the laptop	Windows Movie maker	N/A
Forceps	Vermandel	50.054
Potato dextrose broth	Sigma-Aldrich	P6685-1KG

Comments/Description
Transparent. Length: 3.15 in (8 cm), width: 2.17 in (5.5 cm), depth: 1.77 in (4.5 cm)
3.62x0.63 in (9.2x1.6 cm)
Disposable 1uL. Length: 7.67 in (19.5 cm)
USB Endoscope Camera
Old beaker clamp stand.
Generic laptop for saving recordings.
Soft
24 g/L

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Article Title: A VISUAL GUIDE TO BEH. DEF. TO PATHOGEN ATTACK...

Signature: 

Date: 4/5/18

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Dear Dr. Alisha DSouza

Thank you for the opportunity to submit a revised version of our manuscript JoVE58420 “A visual guide to behavioral defenses to pathogen attack in leaf-cutting ants” by Stephen Nilsson-Møller, Michael Poulsen and Tabitha Innocent for consideration in JoVE.

We appreciate the positive and constructive comments from you and the reviewers and below we outline the changes we have made to the manuscript to accommodate all queries. In addition to these changes, we have made a number of small additional improvements to the manuscript. Line numbers indicated refer to the final revised manuscript without track changes.

We believe that we have addressed all aspects in full and hope the manuscript can be deemed suitable for publication in JoVE.

Thanks in advance for your consideration.

On behalf of all authors,

Kind regards
Michael Poulsen

CC: ndt144@alumni.ku.dk, tabitha.innocent@bio.ku.dk

Dear Dr. Poulsen,

Your manuscript, JoVE58420 A visual guide to behavioral defenses to pathogen attack in leaf-cutting ants, has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300 dpi.

Your revision is due by **Jun 25, 2018**.

To submit a revision, go to the JoVE submission site and log in as an author. You will find your submission under the heading "Submission Needing Revision".

Best,

Alisha DSouza, Ph.D.
Senior Review Editor
JoVE
617.674.1888

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
We have now carefully proof-read the manuscript throughout.
2. Please upload videoclips 1 and 3.
These have now been uploaded as requested.
3. Figure 3: Please change “hrs” to “h”.
Changed as requested
4. Figure 2 legend: Please define SE.
Changed as requested
5. Please rephrase the Introduction to include a clear statement of the overall goal of this method.
We now clearly state the overall goal of this method (Lines 96-100).
6. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.
Checked throughout and changed as requested
7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
Changed as requested.
8. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Changed as requested.
9. Lines 184-299: Please note that information provided here is a better fit for Representative Results than Protocol. Therefore please combine them into the Representative Results section. Note that Representative Results should be explained in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.
This is not a description of an outcome of the protocol, but rather of an essential component in the protocol, which is necessary for readers to correctly use the methods and protocol. The protocol we present is centered on how to observe, identify and define these defensive behaviors in ants, not on how to set up a behavioral experiment per se – methods for which are in widespread use in this field; as such we describe elements of the experimental set-up only as far as they are relevant to being able to observe and describe/identify the behaviors accurately, but these are not the focus. The behavioral identifications, however, are an essential part of the core protocol. We therefore have kept the description as is.
10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
These are highlighted with notes throughout the protocol. To avoid redundancies, we have refrained from further discussion them in the Discussion section.
b) Any modifications and troubleshooting of the technique
This is discussed in Lines 489-93 (and in notes throughout the protocol).
c) Any limitations of the technique
This is discussed in Lines 463-468, 484-491, 493-96.

d) The significance with respect to existing methods
This is discussed Lines 481-487.
e) Any future applications of the technique
This is discussed Lines 396-407, 489-496, 505-508.
11. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
We have uploaded a revised xlsx file with all essential supplies, reagents and equipment.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Firstly, I would like to thank you for the opportunity to review this interesting study on prophylactic behaviour of leaf-cutting ants. Particularly, as a researcher of leaf-cutting ant behaviour for a long time, I consider this study to be indispensable. Although the terminology used to describe ant behaviour is relatively standardized, it is not certain that different researchers are naming the same behavioural act consistently. The proposal of the work is clear and objective and I believe it will be a reference for several studies on ant behaviour.

Major Concerns:
As suggestion, I think the authors should improve the quality of the images exhibiting worker behaviours. It seems to me the plastic cover of the colonies makes the image slightly blurred.
We agree that this would be desirable, but because the behaviors were monitored and filmed as part of an infection experiment, we needed the lids kept on to ensure high humidity for the fungus garden. Since the slight blurriness does not prevent behaviors from being observed, and since many behaviors are so rare that capturing them on video is not trivial, we have retained the existing footage. This is thus a consequence of the inevitable trade-off between resolution and filming sub-colonies for extended periods of time, which we discussed at length to find an optimal solution prior to carrying out the experiment. We resolved this in ways that we believe provide sufficient quality videos while making the protocol accessible and suitable for researchers at large, and have now discussed this in the context of method limitations in the text (Lines 493-99).

Minor Concerns:
Line 46 - Major workers act as soldier in Atta species.
We have removed this specification of soldiers in *Atta*, as it is not essential to the text nor focus of the study.
Line 89 - There are other actinomycete genera isolated from leaf-cutting ants that control Escovopsis too.
We have rephrased this sentence to reflect that *Pseudonocardia* is not the only symbiont protecting against *Escovopsis* (Lines 83-7).
Lines 130-133 - The authors should point out the relevance of the queens in colony behaviour.
This is a good point and we now explicitly address in the manuscript that we expect, but cannot guarantee, that queenless colonies are likely to behave as queenright for the short period of time the experiment ran (Line 133-5).
Line 151 - I would consider inoculating a sterile powder (like graphite) to differentiate between an infection with a fungus and an inert agent.
This would have been an improvement to the protocol, so we suggest this now, although we did not

do it in the experiment in the current manuscript (Line 153-55).
Line 160 - The authors seem confident that this is a bacterium of the genus Pseudonocardia. How?
Previous and recent work indicate that the symbiont population present on the cuticle of *Acromyrmex echinatior* is vastly dominated by *Pseudonocardia* (Poulsen et al. Molecular Ecology 2005; Andersen et al. Molecular Ecology 2013). The presence of other secondarily acquired Actinobacteria is predicted to be likely in addition to, rather than instead of, *Pseudonocardia* (Scheuring & Yu, 2012; Worseley et al., 2018) – and, that any additional Actinobacteria are likely to also play a defensive role, and thus not change the behaviors that are focus of this study. We have clarified this in the text and added suitable additional references (Lines 83-7; 166-9; 463-5; plus above references added throughout).
Line 160 - covering more than
We have rephrased this to 'covering most of the cuticle' (Line 167).

Reviewer #2:
Manuscript Summary:
The introduction is quite good, provides the necessary ant-specific background regarding social immunity, and clearly makes the case for it's protocol.

Major Concerns:
Order of worker introduction into the sub-colonies is unclear and potentially very problematic.
All workers and brood were introduced to sub-colonies at the same time in both control and treatment sub-colonies, and we have edited the protocol in line with the comments below to clarify this (Lines 164-74).

Minor Concerns:
There are several items that need to be addressed:
-In the protocol (B, 1.2) it states that sub-colonies are made 'consisting of 4 minor workers and four major workers' but in B, 1.8 it states that 2 brood are also added. If there is a difference in the demographics of the 0 and 72 hour observations it would require an explanation.
There are no differences in the demographics and we now – due to the comments below – remove the specifications of the demography of ants here to ensure that it is clear that the fungus is added to empty sub-colony containers and that workers and brood are only added later.
-Reading through part B sections 1.1 to 1.3 it seems that the ants are already in the box. But 1.4 to 1.5 indicate that fungus comb is added to an empty box. The order of part B would be better if it stated that comb was added to empty boxes, inoculated and then the ants added (see above comment about demographics). It would also benefit from a brief statement about spore behavior in the absence of ants and how it might influence their behavior.
We agree and have revised accordingly (Lines 142-5, 158-62).
-In addition to the order of operations, I am concerned that there isn't a proper control. While it is important to have the 'spore application control' there is also the basic fact that the ants are removed from their parent colony, transferred, and afforded an opportunity to experience a new setting. This can influence behavior, especially grooming and fungal comb tending, and I would like to see some clarification or assurance that this isn't influencing the results and recorded behaviors.
This is a very relevant point and we now acknowledge in the text that we cannot rule out that behaviors may differ in miniature sub-colonies compared to whole colonies or responses in nature (Lines 170-74, 133-35). This is a general shortcoming of any laboratory experimentation, but does not negate that insights can be obtained. We should also note that we performed the experiment to provide the visual representation of behaviors in the right context (i.e., experimental infection

scenario). We now discuss this explicitly in the manuscript (Lines 481-91).
-There are a couple of points regarding what data was included that require explanation. One is lines 310 - 312. It is unclear what this means. Did you leave out colonies? Are the recordings mainly from 0 hour sub-colonies? Is this why the bottom of figure 3 only has 14 colonies? It is also then curious why the authors feel the need to total the recording time (136hr) because it seems that there isn't this much. These items need clarifying in order for the protocol to have a secure experimental basis and thus application to the broader entomological and ethological communities.
We now clearly state which sub-colonies had to be excluded (Lines 183-6) and the consequences for the specific results section and figures (Lines 320-24).

Reviewer #3:
Manuscript Summary:
In the article "A visual guide to behavioral defenses to pathogen attack in leaf-cutting ants", the authors produced a library of behaviors of leaf-cutting ants related to fungal cultivar cleaning after an infection with a pathogenic fungus. To do so, they made subcolonies from 3 colonies of Acromyrmex echinatior collected in the field. Then they infected half of the subcolonies with a pathogenic fungus of the ants' crop (with two times of infection) and the rest of the subcolonies were kept as controls.
They observed 7 behaviors, which are documented with author produced video clips.
Although they expected to find more cleaning behaviors in infected subcolonies that in the control ones, most statistical analyses gave non-significant results, probably due to the low replication (as mentioned by the authors in the discussion). However, they find tendencies in that direction, with different responses depending on worker size.
I found the clips useful to accurately define the observed behaviors. However I have some comments and suggestions regarding the videos and the methods.

Major Concerns:
-Parts A and B of the protocol (which are not highlighted in yellow) seem to be relevant to include in video clips since are important steps in the article. I think that doing so will also help researchers to perform these assays in an easy and standardized way. In addition, they will help to connect more the methodological and visual parts of the article with the results of the contamination with Escovopsis, which otherwise are not very relevant in the article due to the non-significance of the differences between the treatment and the control. It would also help to show in the video how leaf-cutting colony rearing in performed in the lab, though that may make it too long.
We agree and have therefore highlighted more of the parts A and B protocols (pages 3-4). We have also clarified in the text that our primary aim was to create the catalogue of behaviors for wider use, and that results are merely representative, to demonstrate how the definitions may be used within an experimental set-up (Lines 94-104, 501-508). We will furthermore discuss with the JoVE film crew what options are there for including video documentation of how laboratory colonies and sub-colony are set up.
-Statistics: mixed ANOVA is not clearly explained. Are original colonies IDs included as the mixed variable? the only fixed factor seems to be the treatment vs control, but what about the worker size factor and its interaction with treatment? In addition, why are statistics reported only for some behaviors and not for others? It would be better to statistically test all behaviors separately or with a MANOVA.
To make this clearer, we include a new Table 2, which provides the test results and the overview of factors included in the ANOVAs.

Minor Concerns:
-L. 51-2: this is a relative sentence and no point for comparison was given (ie, they have less diversity regarding other ants? o insects?). In addition, some leaf-cutting ants have genetic variability due to mating with several males (Hughes & Boomsma 2004. Evolution, 58, 1251-1260). In addition, this sentence and those till L. 57 are not relevant to the article and can be deleted or reduced, since authors do not mention the entomopathogenic fungus again.
We agree that this is not crucial for the current study, nor extensively-enough described, so we have removed the sentence and trimmed the introduction to entomopathogenic fungi.
-L. 130: using only 8 workers (4 majors and 4 minors) seems too little for a colony that can contain hundreds of workers. Won't this reduce interactions among workers (ie. allogrooming)?
We now acknowledge that this may affect behavioral responses, and argue for why it is nevertheless a relevant setup (Lines 170-74, and 481-91).
-L. 132: explain why 72 h after infection and not 24h maybe for avoiding the risk of contamination, as mentioned later in the article (L. 310-1).
This is a very good point and waiting 72h certainly poses a risk of contamination/growth of other fungi. However, we chose this time frame to increase the probability that *Escovopsis* would have germinated, as we expected based on unpublished work (Lines 158-62).
-L. 172: why 136h? is not 144h=36 subcolonies*4h? or am I misunderstanding?
We now specify that two sub-colonies (one control and one treatment) after 72h were excluded, due to heavy infections with fungi other than *Escovopsis*, removing 8hrs of footage (Line 183-86).
-For video clips in general: it would be necessary to highlight the focal ant (maybe with an arrow at the beginning of the clip), since there are more than one ant in most clips. In addition, slow motion would help to clearly see some particular behaviors, after they are played at the normal time frame. Video quality (illumination specially) was not good in the videos.
We fully agree and have planned to work with the JoVE staff to encircle or put arrows within the video frames. The limitations our experimental approach placed upon video quality were discussed above.
-L. 184 and 211: video clips 1 and 3 were not available for review.
These have now also been uploaded for review.
-L. 208: this part is not very clear in the video. Maybe you can add another video with high magnification
We agree, but this is again a result of the necessary trade-off between resolution and using multiple cameras for extended periods of recording, and not feasible within this study or approach.
-L. 256-9: this behavior is not recorded in the clip, it will be interesting to also show that behavior
We agree, but this is not possible in the current setup, as distinct waste piles were not visible in the sub-colonies. However, we now specify that this would be a suitable extension of the current protocol (Lines 269-70).
-L. 272-2: this behavior is not recorded in the clip, it will be interesting to also show that behavior
As above, we now specify that this would be a suitable extension of the current protocol (Lines 287-88).
-L. 310-1: I think that the fact that treatment was cross contaminated is not justifying that authors should not include these results since they are investigating the behaviors to fungal infections in general. Thus, the fact that they were contaminated is not justifying not including those results, but maybe discussing that the behaviors at 72h are not caused only by Escovopsis (as was in part mentioned in L. 387-90).
We agree that risk of other fungal infection is possible, and that *Escovopsis* is not the only fungus that could provoke a behavioral response. We have clarified this and the specific sub-colonies that

were excluded from analysis in the protocol and results (Line 183-6, 320-24). We also make clearer our main objective, to create a catalogue of behaviors as a tool for future research, while acknowledging the limitations of the small, illustrative behavioral experiment we use to demonstrate how this might be used in other studies (Line 481-499).

-L. 313: not clear what "timing" means in this context, please, specify

We have rephrased this to ‘time after infection’ (Line 325).

-Table 2: it is redundant with Table 1, and I suggest deleting it

We agree and have merged the previous Tables 1 and 2 into a single new Table 1.

-Figure 3: y axis should say "mean frequency" instead of "mean number"

Changed as requested.

-L. 423-5: not clear what "nest border" means, and the sentence is not very clear. Please, clarify. The same for the next sentence.

We have rephrased this to “edge of the fungus garden” for clarity (Lines 433-37).

-L. 439: differences between Pseudonocardia carring workers and not Pseudonocardia-workers (two of each in every subcolony) were not reported or analyzed. Probably this added noise to statistical results when analyzing grooming. I suggest considering this factor in ANOVAs

We did not distinguish behaviors between these worker groups, because the presence/absence of Actinobacteria was not treated as a factor but merely done to include several worker groups (minors, younger majors, older majors) in the sub-colonies. It could however be included as an extension of the current protocol, which we now specify (Lines 170-74 and 491-93).