Report

Current Biology

Wound-dependent leg amputations to combat infections in an ant society

Highlights

- Ants amputate injured legs of nestmates to improve their survival
- Amputations increase survival for femur injuries but not tibia injuries
- Pathogen spread is slower in femur injuries than the more distal tibia injuries
- Ants are capable of differentiating wound type and adapt their treatment accordingly

Authors

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In brief

Frank et al. provide the first example of the use of amputations to treat infected leg wounds in a non-human animal. The findings demonstrate that ants can adapt their treatment modality depending on wound location.

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Report

Wound-dependent leg amputations to combat infections in an ant society

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SUMMARY

Open wounds pose major infection and mortality risks in animals.[1,2] To reduce these risks, many animal species apply antimicrobial compounds on their wounds.[1-4] Ant societies use antimicrobial secretions from the metapleural gland to combat pathogens,[5-10] but this gland has been lost over evolutionary time in several genera, including *Camponotus*.[11] To understand how infected wounds are handled without the use of antimicrobial secretions from the metapleural gland, we conducted behavioral and microbiological experiments in *Camponotus floridanus*. When we experimentally injured a worker's leg at the femur, nestmates amputated the injured limb by biting the base (trochanter) of the leg until it was severed, thereby significantly increasing survival compared to ants that did not receive amputations. However, when the experimental injury was more distal (at the tibia), nestmates did not amputate the leg and instead directed more wound care to the injury site. Experimental amputations also failed to improve survival in ants with infected tibia injuries unless the leg was amputated immediately after pathogen exposure. Micro-CT scans revealed that the muscles likely responsible for leg hemolymph circulation are predominantly in the femur. Thus, it is likely that femur injuries, by attenuating hemolymph flow, provide sufficient time for workers to perform amputations before pathogen spread. Overall, this study provides the first example of the use of amputations to treat infected individuals in a non-human animal and demonstrates that ants can adapt their type of treatment depending on the location of wounds.

RESULTS AND DISCUSSION

While studying the response of nestmates toward injured individuals in the ant *Camponotus floridanus*, we discovered that injuring the leg of an otherwise healthy worker often led to the amputation of the affected limb by nestmates. Controlled experiments revealed that the response of workers depended on the location of the injury. When ants were injured at the level of the femur, amputation by nestmates occurred in 76% of the cases (n = 17, Figures 1D and S1D). By contrast, amputation never occurred in tibia-injured ants (n = 9, Figures 1D and S1D). Amputation occurred on average 240 ± 50 min after injury (n = 8) and always followed the same pattern. Nestmates would begin licking the wound before moving up the injured limb with their mouthparts until they reached the trochanter. The nestmates then proceeded to repeatedly bite the injured leg until it was cut off (Figures 1A–1C, S1A, and S1B; Video S1).

In the first 3 h after a femur injury, workers spent a similar amount of time performing amputation attempts (6.2 ± 8.8 min, Figure 1B) and wound care behavior (7.8 ± 6.3 min, Figure 1A) on the injured leg (likelihood ratio test: df = 6, Akaike information criterion [AIC] = 3,057.4, χ^2 = 0.99, p = 0.3, n = 8). During wound care, nestmates held the injured limb with their mandibles and front legs, allowing them to lick into the wound for prolonged periods (Figure 1A; Video S2). Both the amount of time spent on amputation attempts and wound care behavior decreased over time during the 3 h after injury (amputation attempts, hierarchical generalized additive model [HGAM]: χ^2 = 8.11, p = 0.004, Figures S1A and S1B; wound care behavior, HGAM: χ^2 = 12.21, p = 0.003, Figures 1E and S1C). Individuals that were amputated at the level of the trochanter by nestmates received significantly less wound care after amputation (39 ± 37 s) than they received after the experimental wound at the femur (468 ± 378 s, HGAM: z = 3.27, p = 0.001, Figure 1E).

To quantify differences in wound care behavior between femur- and tibia-injured ants, we placed both types of ants inside naive sub-colonies and recorded the interactions they received during the first 6 h after injury. Wound care lasted significantly longer on tibia-injured ants (36 ± 10 min) than on femur-injured ants (18 ± 2.7 min, df = 5, AIC = 7,580.3, X^2 = 21.99, p < 0.001,

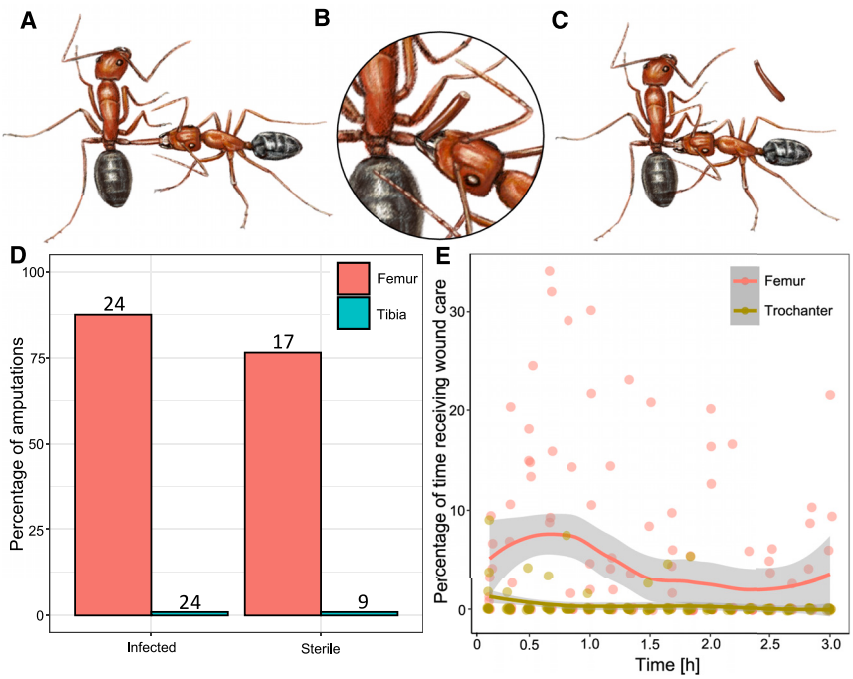

Figure 1. Amputation and wound care behavior in *C. floridanus*

(A) Illustration of a worker providing wound care on a femur-injured individual.

(B) A worker amputating (biting) the injured leg at the trochanter.

(C) A worker providing wound care on the newly created trochanter wound after amputation.

(D) Percentage of amputations performed on ants with an infected or sterile femur (red) or tibia (blue) injury after 24 h. Numbers above the bars represent the sample size for each treatment.

(E) Percentage of time the injured ant received wound care behavior over 3 h, binned in 10 min intervals, with a local polynomial regression (loess) showing a 95% confidence interval for the first 3 h after the experimental femur injury (femur, red: $n = 8$) and the first 3 h after amputation on the trochanter wound (trochanter, brown: $n = 7$). For detailed statistical analyses, see the hierarchical generalized additive models in Figure S1. For videos of the amputation and wound care behavior, see Videos S1 and S2.

$n = 9$, Figure 2). Furthermore, while there was no significant change over time in the frequency of wound care behavior for tibia injuries during the first 6 h after injury (HGAM: $\chi^2 = 2.45$, $p = 0.12$, Figures 2 and S2), wound care behavior decreased rapidly over time for femur injuries (HGAM: $\chi^2 = 79.03$, $p < 0.001$, Figures 2 and S2).

In the termite-hunting ant *Megaponera analis*, individuals with injured legs frequently die from infections unless they are treated by their nestmates, which apply a variety of antimicrobial compounds and proteins secreted from the metapleural gland to infected wounds.[9] Because ants of the genus *Camponotus* do not have a metapleural gland, we hypothesized that amputations might be a way to prevent infections from spreading inside injured individuals. To test this hypothesis, we conducted several experiments. First, we investigated whether *Pseudomonas aeruginosa* is a lethal pathogen in *C. floridanus,* as has been demonstrated in *M. analis*.[9] The application of *P. aeruginosa* (approx. 10^5 bacteria in 10 μL of sterile PBS) on tibia wounds resulted in a 95% mortality rate in 72 h ($n = 48$), a value similar to what had been observed in *M. analis*.[9]

We conducted four treatments to test the effect of wound care and amputation on the survival of individuals, which were injured at the center of either the femur or the tibia. In the first treatment, a sterile PBS solution was applied on the wound and the individuals were isolated (sterile). The second treatment was identical, but the PBS solution contained *P. aeruginosa* (infected). In the third treatment, we also applied a PBS solution containing *P. aeruginosa* but returned the wounded individual to a sub-colony comprising 200 workers. The fourth treatment was identical to the infection treatment, but we additionally cut the ant's leg at the level of the trochanter 1 h after injury to simulate amputation by nestmates (infected + amputated).

For femur-injured individuals, the survival of infected ants in isolation was significantly lower than the survival of ants in the three other treatments (Figure 3A and Table S1). Importantly, there was no significant difference between the three other treatments, showing that our experimental amputations were just as effective as the ones conducted by nestmates in reducing

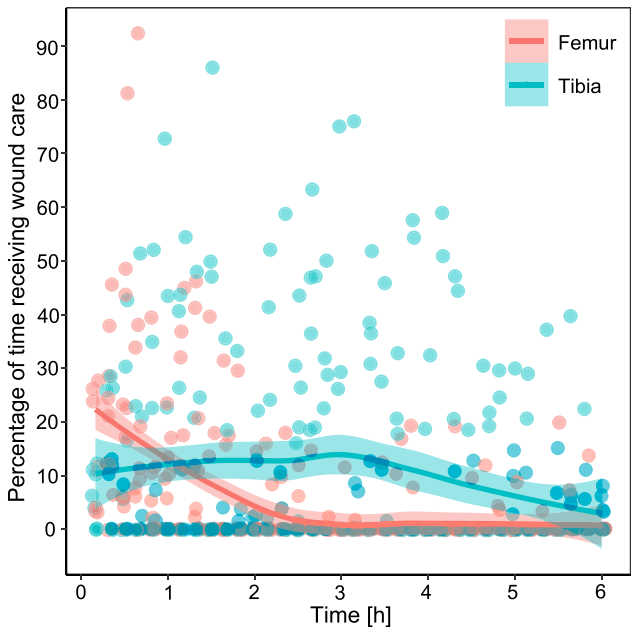

Figure 2. Wound care on femur and tibia injuries

Percentage of time the injured individual received wound care behavior over the first 6 h after injury, binned in 10 min intervals, fitted with a local polynomial regression (loess) showing a 95% confidence interval for femur-injured (red; $n = 9$) and tibia-injured ants (blue; $n = 9$). For detailed statistical analyses, see the hierarchical generalized additive model in Figure S2.

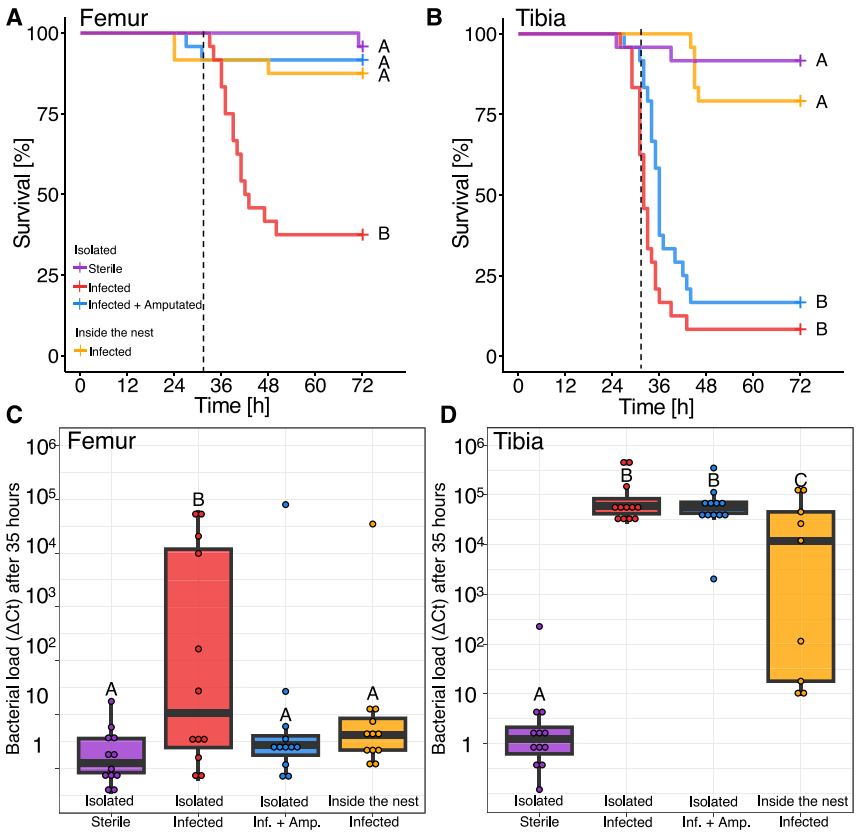

Figure 3. Amputations following femur injuries, but not tibia injuries, increase survival of infected ants

(A) Kaplan-Meier cumulative survival rates of femur-injured ants over 72 h. After cutting the leg at the center of the femur, we conducted four treatments. (1) We exposed the wound to a sterile PBS solution and placed the ants in isolation chambers (purple line). (2) We exposed the wound to a PBS solution containing *P. aeruginosa* and placed the ants in isolation chambers (red line). (3) Same as in (2), but the leg was experimentally amputated at the level of the trochanter 1 h after the wound was infected (blue line, inf. + amp.). (4) Same as in (2), but the ants were returned to their respective sub-colonies after infection (yellow line). Black dashed line: collection time of bacterial load.

(B) Same as in (A) but for tibia-injured ants. $n = 24$ for all treatments. Significant differences ($p < 0.05$) are shown with different letters (detailed statistical results in Table S1).

(C) Boxplots showing ΔCt (bacterial load) values from qPCR amplification of the 16S rRNA gene (normalized against the 28S reference host gene) in ant bodies after removal of the gaster. The ants were subjected to the same treatments as in (A) (n = 12 per treatment). Significant differences ($p < 0.05$) are shown with different letters (detailed statistical results in Table S2).

(D) Same as in (C) but for tibia-injured ants. Box-and-whisker plots show median (horizontal line), interquartile range (box), distance from upper and lower quartiles times 1.5 interquartile range (whiskers), and outliers (>1.5× upper or lower quartile). For further results on the effect of amputation timing on the survival of injured ants see Figure S4.

mortality to a level similar to that of sterile ants in isolation (Figure 3A and Table S1).

For tibia-injured individuals, mortality of infected ants in isolation was significantly higher than for infected femur-injured ants (least square means: Z = −4.36, $p < 0.001$). Otherwise, the results were similar for tibia-injured ants, except for the infected + amputation treatment (Figure 3B). Ants whose legs were experimentally amputated 1 h after infection had a significantly lower survival rate than both sterile ants in isolation (least square means: Z = 4.17, $p < 0.001$) and infected ants returned to a sub-colony (least square means: Z = 4.38, $p < 0.001$, $n = 24$, Figure 3B and Table S1). Importantly, the survival of the experimentally amputated ants was not significantly different from that of the infected ants in isolation (least square means: Z = −2.38, $p = 0.21$, $n = 24$, Figure 3B and Table S1). These results demonstrate that, in contrast to femur injuries, experimental amputation is not effective in reducing mortality of infected tibia-injured individuals (least square means: Z = −4.11, $p < 0.001$, $n = 24$, Figure 3B and Table S1).

These experiments also confirmed our previous finding that workers only amputate the legs of femur-injured ants. Among the individuals that were returned to their colony, none of the 24 tibia-injured ants had their legs amputated, while 21 of the 24 femur-injured ants had their legs amputated (Figure 1D). Interestingly, the three femur-injured ants that did not have their legs amputated died, while all of the 21 individuals whose legs were amputated survived (Figure 3A).

To investigate whether differences in survival among treatments were due to differences in pathogen load, we quantified by qPCRs the pathogen load 35 h after injury and pathogen exposure (Figures 3C and 3D). Across the four treatments, there was a negative association between pathogen load and survival probability both for the femur- (LMER: df = 46, t = 2.97, $p = 0.004$) and tibia-injured individuals (LMER: df = 38.21, t = 9.38, $p < 0.001$).

For femur-injured ants, the pathogen load was highest for the treatment where individuals were isolated after pathogen exposure (Figure 3C and Table S2). By contrast, there were no significant differences among the three other treatments (i.e., sterile in isolation, infected returned to sub-colony, and infected + amputation in isolation, Figure 3C and Table S2). Altogether, these results show that experimental amputation was efficient at reducing the pathogen load of individuals with an infected femur wound.

For tibia-injured ants, the pathogen load was highest for isolated ants with infected wounds, and there was no significant difference between ants whose legs were experimentally amputated or not (t test: t = 0.38, $p = 0.71$, Figure 3D). Individuals that were returned to a sub-colony had an intermediate pathogen load compared to infected ants (with or without amputation) and sterile ants in isolation. Overall, these results show that the pathogen load of infected individuals was decreased by the presence of nestmates that provided wound care but not by experimental amputation.

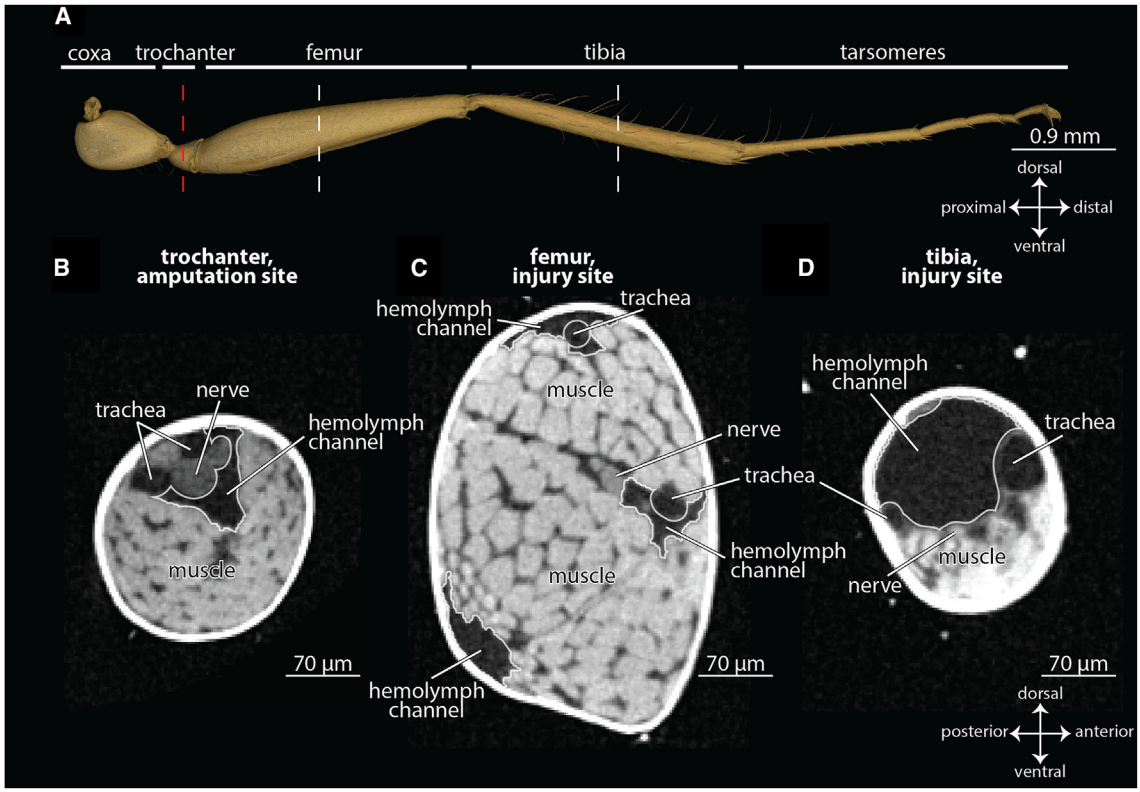

Figure 4. Schematics of the hind leg *of Camponotus floridanus*

(A) A 3D reconstruction of the hind leg. The white dashed lines represent the locations of our experimental femur and tibia injuries, while the red dashed line represents the location where amputations are performed by ants (and by us for experimental amputations).

(B–D) Transverse section of the trochanter (B), femur (C), or tibia (D) injury site.

A possible explanation for the finding that amputation had a positive effect on survival and pathogen load for femur- but not tibia-injured ants is that femur injuries may affect hemolymph circulation differently than tibia injuries. In insects, most of the muscle mass responsible for hemolymph circulation in the leg is located in the femur.[12,13] We confirmed that this is also true in *C. floridanus* by performing detailed micro-CT scan reconstructions of the trochanter, femur, and tibia (Figure 4). The cross sections in the middle of the femur and tibia revealed that the muscle surface was indeed almost 10 times greater in the femur (65,632 μm^2, Figure 4C) than in the tibia (7,554 μm^2, Figure 4D). The destruction of the muscles in femur-injured ants may, therefore, lead to a greater reduction in hemolymph circulation compared to tibia-injured ants. The reconstruction also revealed that the surface of the hemolymph channel is approximately twice as large in the tibia (11,320 μm^2, Figure 4D) as in the femur (5,817 μm^2, Figure 4C). This may further contribute to more pathogens entering the hemolymph circulatory system in tibia injuries than in femur injuries. Consistent with this view, the pathogen load (t test: $t = 6.00$, $p < 0.001$) and the mortality rate (least square means: $Z = -4.36$, $p < 0.001$) were significantly higher in the infected tibia-injured ants than they were in femur-injured ants kept in isolation (Figure 3).

If femur injuries result in less effective hemolymph circulation, it is possible that amputations after 1 h can prevent the pathogen from spreading in the whole body. For tibia injuries, the time required for amputations (which was never less than 40 min, Figure S1B) would be too long to be effective in preventing pathogen spread. To test this hypothesis, we conducted experimental amputations 0, 5, 10, 30, and 60 min after pathogen exposure on isolated ants. These experiments showed that, for tibia-injured ants, experimental amputation was only effective in reducing mortality when performed immediately after pathogen exposure (timepoint 0; Figures S4B and S4D, likelihood ratio test = 17.99, df = 4, $p = 0.001$). By contrast, for femur-injured ants, experimental amputations reduced mortality at all timepoints (Figures S4A and S4C). These results support our hypothesis that the pathogens spread faster in the case of tibia injuries and that amputations at the trochanter are only worth performing for femur injuries.

Our results also reveal that for femur-injured ants, the amputation rate of legs did not differ significantly between ants treated with a sterile solution (76%, $n = 17$) and ants treated with a solution containing *P. aeruginosa* (88%, $n = 24$, Figure 1D, linear mixed model: df = 70, $t = 0.77$, $p = 0.44$). The fact that workers did not behave differently toward infected versus sterile ants contrasts with the finding that in two other ant species, workers discriminate between infected and sterile ants. In the termite-hunting ant *M. analis*, wound care was provided more often to infected individuals than to sterile individuals.[9] Similarly, in *Lasius niger*, workers are able to rapidly detect the presence of a pathogenic fungus[6,7] and adjust their behavior to reinforce the

disease-inhibitory effects of the colony's social network, thus reducing individual contamination risk.[14] A possible explanation for *C. floridanus* workers' amputating the leg of both infected and sterile femur-injured ants is that the risk of infection of injured legs is likely very high under natural conditions. Indeed, experiments in *M. analis* revealed that when experimentally injured ants were kept in isolation on soil from the natural environment, significantly more ants died (80% mortality) than when the soil was sterilized beforehand (25% mortality) or when the wound was treated by nestmates (10% mortality).[8]

In conclusion, we discovered a unique behavior in the animal kingdom to combat infections: workers of the ant species *C. floridanus* amputate the legs of femur-injured ants. Our experiments revealed that this behavior significantly increased the survival probability of the injured ant. By contrast, workers did not amputate the legs of tibia-injured ants. Interestingly, experimental amputation of tibia-injured ants did not increase their survival or reduce the pathogen load in their body. These differences probably stem from the pathogen spreading faster in tibia-injured ants than in femur-injured ants due to morphological differences between the tibia and femur. Irrespective of the reason for amputations being effective in femur-injured but not tibia-injured ants, this study demonstrates that *C. floridanus* possesses the ability to detect the location of wounds and alter their treatment accordingly. Injured legs are only amputated when it increases the survival of the wounded ants (femur injuries); otherwise, nestmates resort to extended wound care sessions (tibia injuries). While humans have conducted medical amputations for over 30,000 years,[15] this is, to the best of our knowledge, the first demonstration that a non-human animal conducts purposeful amputations to improve the survival chances of an injured conspecific.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - *Camponotus floridanus* colonies
  - *Pseudomonas aeruginosa* bacterial growth and preparation
- METHOD DETAILS
  - Wound care behavior in *C. floridanus*
  - Survival of injured ants
  - Bacterial quantification
  - X-ray micro-computed tomography scanning and 3D reconstruction
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2024.06.021.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.T.F.; methodology, E.T.F., D.B., J.L., and L.K.; validation, L.K.; formal analysis, E.T.F. and L.K.; behavioral investigation, E.T.F., D.B., and J.L.; microbial investigation, J.L.; CT-scan analyses, L.A. and E.P.E; resources, L.K.; data curation, E.T.F.; writing – original draft, E.T.F., D.B., and L.K.; writing – review & editing, E.T.F., D.B., J.L., L.A., E.P.E., and L.K.; visualization, E.T.F., D.B., L.A., and J.L.; supervision, E.T.F. and L.K.; project administration, E.T.F. and L.K.; funding acquisition, E.T.F. and L.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Pseudomonas aeruginosa	Frank et al.[9]	N/A
Deposited data		
Raw and analyzed data	This paper	DRYAD REPOSITORY https://doi.org/10.5061/dryad.1rn8pk110
Experimental models: Organisms/strains		
Camponotus floridanus	The Keller laboratory	N/A
Oligonucleotides		
Primer for *P. aeruginosa* (Ps-16S-fw) 5′-GTAGATATAGGAAGGAACACCAG-3′	Frank et al.[9]	N/A
Primer for *P. aeruginosa* (Ps-16S-rv) 5′-GGTATCTAATCCTGTTTGCTCC-3′	Frank et al.[9]	N/A
Primer for normalization based on *Camponotus fellah* (28S-fw) 5′-CTGCTCGGCGGTACGCG-3′	The Keller laboratory	N/A
Primer for normalization based on *Camponotus fellah* (28S-rv) 5′-ACCGGGGACGGCGCAAA-3′	The Keller laboratory	N/A
Software and algorithms		
RStudio	The R Foundation	https://www.r-project.org
R-code	This paper	Zenodo Repository https://doi.org/10.5281/zenodo.11185799
Amira 2019.2	Thermo Fisher scientific	https://www.thermofisher.com/de/de/home/electron-microscopy/products/software-em-3d-vis/amira-software/cell-biology.html?cid=msd_vds_ls_none_amr_123456_gl_pso_gaw_escchz&gad_source=1&gclid=CjwKCAjwmYCzBhA6EiwAxFwfgOsDwwNHKiJomWwxe11fm1zzG8XaVxL5xereX4r41GfbgOWNDYyBJRoCsJoQAvD_BwE
Zeiss Scout-and-Scan Control System	Zeiss	https://www.zeiss.com/microscopy/us/l/campaigns/scout-and-scan.html
VGStudio 2022.1	Volume Graphics GmbH	https://www.volumegraphics.com/en/products/vgstudio.html
Other		
Zeiss Xradia 510 Versa 3D X-ray microscope	Frank et al.[9]	https://www.zeiss.com/microscopy/en/products/x-ray-microscopy/xradia-versa.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Erik T. Frank (erik.frank@uni-wuerzburg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper is available in the Dryad repository (https://doi.org/10.5061/dryad.1rn8pk110).
- All code developed in this paper is available in the Zenodo repository (https://doi.org/10.5281/zenodo.11185799).
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Camponotus floridanus colonies

Colonies were reared from queens collected in 2017 (Florida, United States) and kept at the University of Lausanne in a climatized room at 26°C with 65% humidity and a 12h day/night cycle. Although workers of *C. floridanus* show a continuous size distribution, there are two clear recognizable castes, minors (length = 5.5-7mm, head width < 1.5mm) and majors (length = 8-10mm, head width > 2.7mm).[16] Ants were kept for one week in experimental boxes (17.5 × 23cm) for acclimatization before the experiments. We used only minor workers in the experiments.

Pseudomonas aeruginosa bacterial growth and preparation

The *P. aeruginosa* strain used to infect the wounds of injured ants was isolated from surface soil samples collected in 2018 in the Comoé National Park (Côte d'Ivoire) as part of a previous study by Frank et al. 2023.[9] For the preparation of the pathogen sample, we plated *P. aeruginosa* from a frozen stock (kept in tryptic soy broth medium with 25% glycerol) on tryptic soy agar plates. After 24 h at 30°C the bacterial culture was replated and left to grow for an additional 24 h. The pathogen was then diluted in a sterile phosphate-buffered saline (PBS) solution to approx. 10^5 bacteria in 10 μL of PBS (optical density = 0.005) using a portable Ultrospec 10 cell density meter from Biochrom.

METHOD DETAILS

Wound care behavior in *C. floridanus*

Focal individuals were color tagged with acrylic paint two days before each experiment. In a first experiment, we compared the wound care behaviors towards ants with a sterile femur injury and their subsequent injury after amputation of the injured leg by nestmates at the trochanter. In each of four sub-colonies (each containing 47 foragers, 50 nurses and 3 majors but no brood or queen), we placed two ants whose right hind legs were cut at the center of the femur (Figure 4A). All experimental injuries were done with a sterile Dowel-scissor (Fine Science Tools). Before cutting the leg, the focal individuals were cooled for 2 min by placing them in a glass container surrounded by ice. This allowed easier manipulation and precision during the cut, which was always located at the center of the tibia or femur. Each sub-colony was placed in a climate box and filmed with an infrared camera for 24 h after injury. We then quantified the first 3 h after injury and the first 3 h after amputation.

In a second experiment, we compared the wound care behaviors toward injured nestmates with either a sterile wound at the level of the femur or tibia. Ants' right hind legs were cut either at the center of the femur (n = 10) or center of the tibia (n = 10). Two femur-injured and two tibia-injured individuals were then returned in each of five naive sub-colonies containing 95 foragers, 100 nurses and 5 majors (Figure 2). There were thus two femur- and two tibia-injured ants inside each sub-colony at the same time. Because one femur- and one tibia-injured ant lost the color ID, sample sizes are n = 9 per treatment. Sub-colonies were filmed with an infrared camera in climate boxes for 1 h before the treatments and for the subsequent 6 h. To maintain the ratio of healthy to injured ants the same across experiments, we had to double the number of individuals in each sub-colony in the second experiment (from 100 ants in Figure 1, to 200 ants in Figure 2). Both experiments were conducted in November and December 2020.

Behavioral analyses of the videos were performed using VLC media player v. 3.0.11-win64 with the help of the add-on Zoomit. Wound care behaviors were classified into four categories: (1) ''femur wound care'' when nestmates groomed/licked the subject at the experimental femur wound before amputation. (2) ''Tibia wound care'' when nestmates groomed/licked the subject at the experimental tibia wound. (3) ''Trochanter wound care'' when nestmates groomed/licked the subject at the trochanter wound after amputation and (4) **"**amputation'' when nestmates bit the injured leg on the trochanter.

Survival of injured ants

We conducted four treatments to test the effect of wound care and amputation on the survival of individuals injured either at the center of the femur or tibia. In the first treatment, a sterile PBS solution was applied on the wound and the individuals were isolated. The second treatment was identical, but the PBS solution contained *P. aeruginosa.* In the third treatment, we also applied a PBS solution containing *P. aeruginosa* but returned the wounded individual to a sub-colony comprising 200 workers. Finally, the fourth treatment was identical to the second treatment, but we experimentally amputated the injured leg with sterile microscissors at the level of the trochanter 1 h after injury. The four treatments were conducted both for ants injured at the level of the femur or tibia. Six ants from each of the six sub-colonies were used per treatment, making a total of 36 ants per treatment, 24 of which were used for the survival analysis (n = 24) and 12 were collected after 35h for the qPCR analyses (outlined in the next section; n = 12). In all treatments, ants had access to food (honey water) and water. For treatments 2-4, injuries were infected by exposing the injury to a 10 μL solution of gram-negative bacteria (*P. aeruginosa*) diluted in sterile PBS (approx. 10^5 bacteria in 10μL of PBS) following the same protocol and pathogen strain of Frank et al. 2023. For treatment 1 ants had their wound exposed to a sterile solution of PBS. Manipulated ants were checked once per hour for the next 48 h and once every 2 h from 48 to 72 h.

To assess the impact of the delay between pathogen exposure and experimental amputation on survival for femur- and tibia-injured ants, we repeated the same survival experiment as before but with five different amputation times: 0, 5, 10, 30 and 60 min after pathogen exposure (Figure S4). Each treatment included 12 replicates, each from a different colony. One replicate had to be removed due to a humidity malfunction in one of the systems and four individuals were removed from the analysis because they

died before the survival recordings began due to handling mistakes. This second survival experiment was conducted in December 2023.

Bacterial quantification

To compare the proliferation of bacteria in infected ants kept in isolation or inside sub-colonies, we performed qPCR analyses 35 h after manipulation on a third of the individuals from the survival experiment (n = 12 for each of the 8 treatments; Figures 3C and 3D). The gasters of the focal individuals were removed to reduce potential noise from the gut bacteria.

DNA was extracted from the bodies by snap-freezing them in liquid nitrogen for better homogenization with a Precellys Evolution homogenizer (Bertin Technologies) at 6500 rpm for 2 × 30 s using a pool of zirconium ceramic beads. We then added 180 μL of ATL buffer and 20 μL of proteinase K (20 mg mL^{-1}) and digested these homogenates at 56 °C overnight, after which DNA was extracted using a Qiagen BioSprint 96 robot with the BioSprint DNA Blood Kit following the manufacturer's instructions. Bacterial loads were quantified with a QuantStudio qPCR instrument (Applied Biosystems) using thermal cycling conditions as recommended for SYBR Select Master Mix and using the protocols published in Kešnerová et al.[17]

To calculate *P. aeruginosa* bacterial loads, we used primers Ps-16S-fw 5′-GTAGATATAGGAAGGAACACCAG-3′ and Ps-16S-rv 5′-GGTATCTAATCCTGTTTGCTCC-3′, which were originally developed for Frank et al. 2023,[9] and for normalization we targeted the *C. fellah* 28S gene with primers 28S-fw 5′-CTGCTCGGCGGTACGCG-3′ and 28S-rv 5′-ACCGGGGACGGCGCAAA-3'. *P. aeruginosa* 16S rRNA gene copy numbers (target gene) were expressed relatively to *C. fellah* 28S rRNA reference gene copy numbers based on the Pfaffl method[18] using the equation: $\Delta Ct = (Etarget)^{\Delta Ct,\ target\ (calibrator\ -\ test)}/(Eref)^{\Delta Ct,\ ref\ (calibrator\ -\ test)}$, where Etarget is the amplification efficiency (calculated based on a 10X serial dilution) of the *Pseudomonas* 16S rRNA gene (Etarget = 2), Eref is the amplification efficiency of the 28S *C. fellah* reference gene (Eref = 1.827); ΔCt, ref (calibrator - test) is the Ct of the reference gene in a sample used as calibrator (sample 13) minus the Ct of the reference gene in the test sample; ΔCt target (calibrator - test) is the Ct of the target gene in the calibrator minus the Ct of the target gene in the test sample.

X-ray micro-computed tomography scanning and 3D reconstruction

To examine the internal structure of the leg at the amputation and injury sites, the hind leg of a *C. floridanus* worker (specimen was stored in ethanol 100%; unique specimen identifier: CASENT0741352) was scanned (Figure 4). To obtain a higher resolution scan, the hind leg was detached from the body, stained in 2M iodine for seven days and scanned using Zeiss Xradia 510 Versa 3D X-ray microscope at the Okinawa Institute of Science and Technology Graduate University, Japan, and reconstructed using Zeiss Scout-and-Scan Control System software (version 16.1.14271.44713). The scanning parameters were 40 kV/3 W beam strength with 4s exposure under a 4x magnification, −11mm source distance, and 2001 projections, which resulted in a voxel size of 2.25μm. The resulting scan was segmented using Amira 2019.2 (Thermo Fisher Scientific, Berlin, Germany), the cross-sections were visualized in Amira, and the rendering of the hind leg was visualized using VGSTUDIO 2022.1 (Volume Graphics GmbH, Heidelberg, Germany).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses and graphical illustrations were performed using the statistical software R v.4.0.3 with the interface Rstudio v.1.3.1093 and the R packages lme4, mgcv, ggplot2, ggtext, DHARMa, survival and survminer. For all analyses, the worker ID was kept as a random effect nested within the sub-colony effect. To compare amputation rates between tibia- and femur-injured ants with and without infections, we conducted a hierarchical Bayesian model (Figure S1D). The model was fitted using Stan accessed via brms (v.2.21.0) - see supplemental code in the dryad repository for details on the model. For behavioral differences in wound care between sterile and infected individuals (Figures S1 and S2), we modeled wound care as a binary event using binomial generalized additive models (HGAM) with post-hoc contrasts to identify binned intervals of time during which the probability of receiving wound care differed between sterile and infected individuals. Assumptions of the HGAM models were graphically verified using DHARMa in Figure S3. To test for significant differences in survival (Figures 3A, 3B, and S4, Table S1), we conducted mixed-effect Cox proportional hazards regression models using the R package Survminer (v.0.4.9). For post-hoc analyses of the models, least square means were compared using the R package lsmeans (v.2.30; Table S1). For differences in bacterial load, we conducted a restricted maximum likelihood linear mixed model followed by a post-hoc analysis with least square means differences with two-tailed Student's t test and a Holm-Bonferroni correction using the software JMP v.15.1.0 (Figures 3C and 3D, Table S2).