Bacterial exposure leads to variable mortality but not a measurable increase in surface antimicrobials across ant species

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Social insects have co-existed with microbial species for millions of years and have evolved a diversity of collective defenses, including the use of antimicrobials. While many studies have revealed strategies that ants use against microbial entomopathogens, and several have shown ant-produced compounds inhibit environmental bacterial growth, few studies have tested whether exposure to environmental bacteria represents a health threat to ants. We compare four ant species' responses to exposure to *Escherichia coli* and *Staphylococcus epidermidis* bacteria in order to broaden our understanding of microbial health-threats to ants and their ability to defend against them. In a first experiment, we measure worker mortality of *Solenopsis invicta*, *Brachymyrmex chinensis*, *Aphaenogaster rudis*, and *Dorymyrmex bureni* in response to exposure to *E. coli* and *S. epidermidis*. We found that exposure to *E. coli* was lethal for *S. invicta* and *D. bureni*, while all other effects of exposure were not different from experimental controls. In a second experiment, we compared the antimicrobial ability of surface extracts from bacteria-exposed and non-exposed *S. invicta* and *B. chinensis* worker ants, to see if exposure to *E. coli* or *S. epidermidis* led to an increase in antimicrobial compounds. We found no difference in the inhibitory effects from either treatment group in either species. Our results demonstrate the susceptibility to bacteria is varied across ant species. This variation may correlate with an ant species' use of surface antimicrobials, as we found significant mortality effects in species which also were producing antimicrobials. Further exploration of a wide range of both bacteria and ant species is likely to reveal unique and nuanced antimicrobial strategies and deepen our understanding of how ant societies respond to microbial health threats.

1 **Bacterial exposure leads to variable mortality but not**

2 **a measurable increase in surface antimicrobials**

3 **across ant species**

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16 **Abstract**

18   Social insects have co-existed with microbial species for millions of years and have

19   evolved a diversity of collective defenses, including the use of antimicrobials. While many

20   studies have revealed strategies that ants use against microbial entomopathogens, and several

21   have shown ant-produced compounds inhibit environmental bacterial growth, few studies have

22   tested whether exposure to environmental bacteria represents a health threat to ants. We compare

23   four ant species' responses to exposure to *Escherichia coli* and *Staphylococcus epidermidis*

24   bacteria in order to broaden our understanding of microbial health-threats to ants and their ability

25   to defend against them. In a first experiment, we measure worker mortality of *Solenopsis invicta*,

26   *Brachymyrmex chinensis*, *Aphaenogaster rudis*, and *Dorymyrmex bureni* in response to exposure

27   to *E. coli* and *S. epidermidis*. We found that exposure to *E. coli* was lethal for *S. invicta* and *D.*

28   *bureni*, while all other effects of exposure were not different from experimental controls. In a

29   second experiment, we compared the antimicrobial ability of surface extracts from bacteria-

30   exposed and non-exposed *S. invicta* and *B. chinensis* worker ants, to see if exposure to *E. coli* or

31   *S. epidermidis* led to an increase in antimicrobial compounds. We found no difference in the

32   inhibitory effects from either treatment group in either species. Our results demonstrate the

33   susceptibility to bacteria is varied across ant species. This variation may correlate with an ant

34   species' use of surface antimicrobials, as we found significant mortality effects in species which

35   also were producing antimicrobials. Further exploration of a wide range of both bacteria and ant

36   species is likely to reveal unique and nuanced antimicrobial strategies and deepen our

37   understanding of how ant societies respond to microbial health threats.

Introduction

38

39   Organisms that live in societies, whether they are ants, termites or humans, benefit from

40   cooperative defense, offspring care, and foraging success. However, high levels of relatedness in

41   some social species, combined with high densities of individuals, whether in big ant colonies or

42   big human cities, can cause greater susceptibility to pathogens and parasites (Hughes, Eilenberg

43   & Boomsma, 2002; Sengupta, Chattopadhyay & Grossart, 2013; Schmid-Hempel, 1998). Human

44   societies reduce pathogenic and parasitic loads in many ways, in particular, antibiotics have

45   saved millions of lives (Levy, 1992). Like humans, ants also have hygienic behaviors that reduce

46   transmission (Konrad et al., 2018), can employ vaccination-like behavior (Konrad et al., 2012),

47   and employ a variety of antimicrobials (Schlüns & Crozier, 2009). For social insects in general,

48   and ants in particular, a first step in understanding antimicrobial strategies is to assess which

49   microbes present health threats and how responses to microbial exposure might vary.

50   Extant social insect taxa have co-existed with microbial species for millions of years and

51   have evolved a diversity of collective defenses (Meunier, 2015). These defenses include hygienic

52   behaviors such as allogrooming—the grooming of nest-mates—or the removal of waste and dead

53   individuals (Cremer, Armitage & Schmid-Hempel, 2007). Ants and other social insects also

54   produce prophylactic antimicrobial compounds (Traniello, Rosengaus & Savoie, 2002; Yek &

55   Mueller, 2011). In ants, prophylactic antimicrobial compounds are most well-documented in

56   response to fungal entomopathogens (Schlüns & Crozier, 2009). However, the microbial-rich

57   environments of ground-nesting ants facilitates interactions with many more species of bacteria

58   than fungi, and many of these bacteria seem likely to have the potential to kill ants, even if they

59   are not specialized entomopathogens (Hoggard et al., 2013).

60   How harmful exposure to non-entomopathogenic bacteria is to ants is largely unknown.

61   However, many studies have demonstrated that compounds produced by ants are effective at

62   inhibiting growth of non-entomopathogens. For example, one early study showed inhibition of

63   the gram-negative bacterium *E. coli* (Mackintosh et al., 1995) by peptides synthesized by the

64   metapleural glands of the ant *Myrmecia gulosa* (Fabricius, 1775). Concentrations of metplueral

65   gland secretions of at 10-ant equivalents had a 99% kill rate on microbial cells. Metaplueral

66   gland peptides can disrupt the cell walls of gram-negative bacteria (*E. coli*) as well as plasma

67   membranes of gram-positive bacteria (*Bacillus cereus*) (Mackintosh et al., 1995). The venom

68   from *Solenopsis invicta (*Buren, 1972) contains multiple alkaloids that have different levels of

69   antimicrobial effectiveness against gram-negative and gram-positive bacteria (Blum et al., 1958),

70   with a generally higher ability to inhibit gram-positive bacteria (Jouvenaz, Blum & MacConnell,

71   1972). In another study, *S. invicta* venom alkaloids were isolated and found to be effective at

72   inhibiting *Pseudomonas fluorescens* biofilms (Carvalho et al., 2019). Finally, a comparison of

73   surface extracts from 20 ant species from four subfamilies found that extracts from 40% of tested

74   species were ineffective at inhibiting the growth of *Staphylococcus epidermidis* at just a 5-ant

75   extract equivalent (Penick et al., 2018). That comparative study as well as others on wasps

76   (Hoggard et al., 2011; 2013), bees (Stow et al., 2007), and thrips (Turnbull et al., 2010) draw

77   evolutionary conclusions about these organisms' antimicrobial activity by assessing their ability

78   to inhibit a non-entomopathogenic bacterial species (usually *Staphylococcus spp.*). However,

79   although ant-produced compounds are capable of inhibiting these bacteria, we do not know

80   whether exposure to non-entomopathogenic and unfamiliar bacteria represent an actual health

81   threat that ants would benefit from being able to defend against.

82   Exposure to pathogens often produces physiological immune responses, where low-risk

83   challenges of pathogens upregulate individual and group immunity. In a study with the ant

84   *Formica exsecta* Nylander, 1846, workers were orally exposed to *Serratia marcescens, E. coli,*

85   or *Pseudomonas entomophila* (Stucki et al., 2019). Antimicrobial gene expression of ants

86   exposed to *P. entomophila* and *E. coli* was upregulated after exposure, possibly as a general

87   immune response from exposure to a high bacterial load or as the ant's prophylactic response.

88   The response of *F. exsecta* suggested that even non-entomopathogenic bacteria (*E. coli*) can

89   provoke immune or antimicrobial responses from ants. However, extrapolation of results from a

90   few species of ants to general patterns are likely to mislead, as ants are a diverse group of

91   organisms with variable life history traits that likely result in different strategies for dealing with

92   microbial exposure. Comparative studies across ant species are likely to reveal key differences in

93   antimicrobial strategies.

94   Recently, the lethality of fungal pathogen exposure was compared across 12 species of

95   ants from 4 genera (Bos et al., 2019). The study found species-level mortality differed across,

96   and even within, ant genera in response to identical pathogen exposure. In addition to this, the

97   three mostly closely related species tested had the most similar responses, suggesting disease

98   resistance might be phylogenetically linked traits. Similar conclusions were reached in a study of

99   surface antimicrobials collected from 20 species of ants (Penick et al., 2018). The antimicrobial

100   ability, measured through inhibition of *S. epidermidis* cultures, using ant-derived surface extracts

101   was highly variable across species; however, there was a phylogenetic signal associated with

102   species that yielded inhibitory extracts.

103   In this study, we consider four ant species (*Solenopsis invicta*, *Brachyponera chinensis*

104   (Emery, 1895), *Aphaenogaster rudis* Enzmann, 1947, and *Dorymyrmex bureni* (Trager, 1988))

105   whose surface antimicrobials have been shown to be differently effective at inhibiting the

106   human-associated bacteria species, *Staphylococcus epidermidis* (Penick et al., 2018). In Penick

107   et al. (2018), surface extracts from *S. invicta* and *D. bureni* workers were very effective at

108   inhibiting growth of *S. epidermidis*, whereas *A. rudis* and *B. chinensis* ants showed weak

109   inhibitory ability. These results and the above referenced pharmacological work indicating that

110   some ant-derived compounds effectively inhibit the growth of *S. epidermidis* and *E. coli* bacteria

111   have led us to the research question of whether non-ant associated bacteria represent an actual

112   health threat to different ant species. Perhaps ants which are susceptible to exposure to a broad

113   range of bacteria produce broadly effective antimicrobials as a compensatory strategy. Therefore,

114   we first compare the lethality of bacterial exposure across those four ant species to a gram-

115   positive bacterium, *S. epidermidis*, and a gram-negative bacterium, *E. coli*. We do this by

116   exposing live groups of ant workers with agar-grown bacteria and measuring their mortality over

117   48 hours.

118   In a second experiment, we tested if exposure to bacteria under these conditions resulted

119   in an increase in antimicrobial compounds on the bodies of two of these species (*S. invicta* and

120   *B. chinensis*). In a previous study, surface extracts from *S. invicta* were strong inhibitors of

121   bacterial growth, and *B. chinensis* lacked inhibitory ability (Penick et al., 2018). One possible

122   explanation for this difference is that some species may deploy antimicrobial compounds only in

123   response to exposure to harmful microbes. We test this by extracting surface compounds from

124   two species of bacteria-exposed ants and testing their bacterial inhibitory ability as compared to a

125   control. We predicted that exposure to a potentially lethal bacterial challenge would result in an

126   increase of antimicrobial ability from ant surface extracts, which would indicate that the

127 antimicrobial abilities of some ants are able to be conditionally deployed to meet direct microbial

128 challenges.

129

Materials & Methods

131   Bacteria were sourced from Carolina Biological Supply Company. *Staphylococcus*

132 *epidermidis* and *Escherichia coli* strain K12 were kept in glycerol stocks at -80 °C, then spread

133 onto BD Difco™ LB, Miller (Fisher Scientific) agar 100 mm x 15 mm petri dish plates. Plates

134 were incubated for 24 h at 35 °C. Bacteria were cultured on plates for experiment 1 at 0.5

135 MacFarland standard. For experiment 2, bacteria from agar plates were also cultured in liquid

136 BD Difco™ LB, Miller media. Liquid cultures were incubated for 24 h at 35°C before being

137 used in assays.

Experiment 1: Ant mortality in response to exposure to bacteria

139   Worker mortality in response to bacterial exposure was measured across four ant species.

140 Fire ants, *Solenopsis invicta*, were collected between June-December of 2018 in Raleigh, NC,

141 USA by collecting the tops of mounds with a shovel. *Aphaenogaster rudis* group ants were

142 collected in Durham, NC, USA and Raleigh, NC from July to August of 2018 by aspirating

143 workers from within their nests. Asian needle ants, *Brachyponera chinensis*, were collected in

144 Raleigh, NC in December 2018 to May 2019 by opening dead logs to expose workers for

145 collection via aspiration. *Dorymyrmex bureni* colonies were collected in Hoffman, NC, USA in

146 June 2019 by collecting mounds with a shovel. Species used in this and the following experiment

147 were identified using morphological keys (MacGown, 2014), and voucher specimens are in the

148 collection of author AAS at the North Carolina Museum of Natural Sciences. Each species of

149 ants was separated from its nesting material to reduce further environmental (microbial)

150   interaction and kept in the lab for <24 h before experimentation. Before experimentation, while

151   in the lab, ants were given a supply of 20% sugar water (Kay et al., 2014).

152   In each exposure treatment, we used 200 *S. invicta* workers and 50 *B. chinensis*, *D.*

153   *bureni*, and *A. rudis* workers (Fig. S1). The difference in number of workers used for *S. invicta*

154   was based on what could be consistently captured from colonies. Experiments with each species

155   were replicated across 15 colonies, where each colony was subject to three treatments.

156   Treatments were: exposure to *E. coli*, exposure to *S. epidermidis*, or exposure to sterile agar as

157   control. Exposure experiments were performed in lid-covered deep petri dishes (100mm x 25

158   mm). The dishes were prepared with Insect-A-Slip (BioQuip Products) applied around the

159   interior edge to prevent ant escape. Ants were exposed to a small piece of agar (10 mm x 9.7cm^2)

160   that had a bacterial lawn of *S. epidermidis*, *E. coli*, or no bacterial growth as a control. Bacterial

161   lawns were grown from liquid culture incubated overnight at 35 °C in a shaking incubator

162   adjusted to a 0.5 MacFarland standard. In the experimental arena, agar pieces with and without

163   bacteria were hydrated with 100 μl of MilliQ H$_2$O, this prevented agar from drying out and

164   encouraged ant interaction with agar. Depending on the species being tested, all 200 or 50 ant

165   workers were introduced at once to the experimental arena after water was absorbed by agar by

166   directly placing ants on agar to ensure at least one point of direct exposure. Mortality was

167   measured by counting dead ants at 24 and 48 h. Observations were recorded when ants tunneled

168   into agar, as an additional indication of direct interaction with the bacteria.

169   We tested the mortality effect of exposing a group of workers as compared to workers

170   from the same colony exposed to control, non-bacterial, treatments. Data at 24 h and 48 h were

171   graphed and analyzed with a Friedman's rank sum test with Finner post hoc pairwise

172   comparison. Friedman's test is similar to a one-way ANOVA for an unreplicated complete

173   blocked design of nonparametric data (García et al., 2010). The Finner post hoc comparison

174   procedure was used for indicating significance of treatment effects. Finner's post hoc uses a step-

175   down p-value adjustment value; it rejects test statistics when $p_i > 1-(1-a)^{(k-1)/I}$ (García et al.,

176   2010). Friedman's statistical tests used at a significance level of $\alpha = 0.05$, and Finner post hoc

177   used an adjusted threshold level of significance of $\alpha_1 = 0.0975$, $\alpha_2 = 0.05$, or $a_3 = 0.0336$.

178   Analysis was carried out in R version 3.5.2 (R Core Team, 2017) using the packages scmamp

179   (Calvo & Santafe, 2016) and devtools (R Core Team, 2017).

180   **Experiment 2: Does exposure to bacteria lead to an increased presence of surface**

181   **antimicrobials?**

182   We compared the antimicrobial ability of surface extracts from bacteria-exposed and non-

183   exposed *S. invicta* and *B. chinensis* worker ants. We wanted to know if antimicrobial activity of

184   surface extracts was a constant feature of these species, or something that might be responsive

185   and increasing when exposed to bacterial threats. For the experiment, we collected *B. chinensis*

186   ants by opening wood logs with their nests and aspirating workers. The ants were collected

187   between January and May 2019. To collect *S. invicta* ants, the tops of nest mounds were

188   removed, and workers were aspirated from removed soil. Ants were collected between June and

189   December 2018. Both species were collected in Raleigh, NC.

190   The experiment consisted of an exposure treatment followed by extracting surface

191   compounds of workers to be challenged by bacteria in a liquid culture well plate assay (Fig. S2).

192   As was done for experiment 1, 200 *S. invicta* workers and 50 *B. chinensis* workers were used for

193   each exposure treatment. We used 15 colonies per species for treatments, in an incomplete block

194   design. Treatments were exposure to *S. epidermidis*, *E. coli*, or sterile agar for control, following

195   the protocol used for experiment 1 described above. Bacterial lawns were grown from liquid

196   bacterial cultures of *E. coli* and *S. epidermidis* adjusted to a 0.5 MacFarland standard after

197   growing overnight at 35 °C in a shaking incubator. 100 μl of MilliQ H2O was pipetted under the

198   agar piece to hydrate the media and encourage ants to interact with the agar plugs. Exposure

199   treatments ran for 6 h. Then, any dead individuals were removed from exposure before freeze-

200   killing the remaining living workers for extraction. The 6 h exposure time was determined from

201   earlier trials and selected as a, largely, sub-lethal level of exposure as lethal effect of exposure

202   were first seen after 6 h.

203   Surface compounds of 40 workers from each exposure treatment were extracted in 360 μl

204   isopropanol for 15 min after an initial vortex spin for 15 s. Whole workers were used, and ant

205   bodies remained in-tact through the vortexing and solvent extraction. Previous studies have

206   shown effectiveness of using polar solvents (e.g. ethanol) to extract antimicrobial compounds

207   (Penick et al., 2018; Stow et al., 2007; Turnbull et al., 2011). Extracts were filtered through 0.2

208   micron SEP filters (Stow et al., 2007). Isopropanol extracts were evaporated in a vacuum

209   centrifuge then resuspended in 360 μl LB media.

210   We adjusted a 96 well plate assay protocol for testing antimicrobial ability against

211   bacterial liquid culture (Stow et al., 2007). The initial 40-worker-ant extract was divided into 4

212   equal parts; 10 ant equivalents of ant body-surface extracts (90 μl) were tested against 100

213   CFU's of either *S. epidermidis* or *E. coli*. Bacterial controls of 100 CFU's bacteria (*S.*

214   *epidermidis* or *E. coli*) with LB media were plated as maximum growth controls. Another 10 ant

215   equivalent extracts suspended in LB media were plated as minimum growth controls. Media

216   controls were also plated to verify there was no contamination. Plates were incubated at 35 °C

217   for 18 h before pipetting 10 μl of WST-8 (PromoKine, Heidelberg, Germany) followed by

218   another hour of incubation to allow the salt to bind to available live cells. WST-8 salt binds to

219 cellular membranes of organisms that are undergoing active transport, and therefore, provides a

220 colorimetric method to analyze live microbial count in culture (Braun et al., 2018). After

221 incubation, optical density was measured at OD_{600} with 5 s shaking before reading using a plate

222 reader (SpectraMax M5, Molecular Devices, USA).

223   OD_{600} readings of the well plate were comparable values across the plate because of

224 WST-8 binding equally to available live cells (PromoKine, Heidelberg, Germany). Readings of

225 exposure treatment workers were measured as percent inhibition adjusted relative to the control

226 treatment workers. Antimicrobial ability was determined by comparing adjusted percent

227 inhibition to plated maximum and minimum growth controls. Final results were reported as

228 change in percentage of inhibition of microbes between treatment and control groups. Results

229 provided either a positive or negative inhibition value based on the treatment inhibition relative

230 to the control.

231   In our results some of the outlying data points are percent inhibition measures beyond

232 100% and below 0%. Each data point is a measure of inhibition in wells where half of the ant

233 extract is added to an experimental well with live bacteria; however, we report this measure

234 relative to two other wells: one containing only bacteria and media (a maximum bacterial growth

235 control) and the second containing only media and the other half of the ant extract (a minimum

236 growth control). For the outlying points beyond 100%, the half of the ant extract used as

237 minimum growth control had a higher absorbency reading (the proxy for bacterial growth) than

238 the other half of the ant extract used in the experimental well. Minimum growth control wells

239 could have higher absorbency readings from WST-8 binding to available molecules present in

240 the extract, such as carbohydrates or even secondary products that could be contributing to the

241 antimicrobial ability seen in the experimental well. For the outlying points below 0%, the

242   experimental well absorbency was higher than the maximum growth control. This was likely due

243   to the ant extract failing to inhibit the microbial challenge in the experimental well, and instead

244   supplementing the growth of the bacteria culture.

245   We tested if antimicrobial ability showed a conditional response with a Kruskal Wallis

246   rank sum test. The Kruskal Wallis analysis tests non-parametric data for multiple groups when

247   assumptions for ANOVA are not met—in our case, our data was not a complete design. Some

248   replicates were comprised of samples from two nests to complete all three treatments. In these

249   instances, a control and single treatment group were sampled from colonies twice—once for *E.*

250   *coli* and once for *S. epidermidis*. All statistical tests used a threshold level of significance of α =

251   0.05. Analysis was carried out in R version 3.5.2 (R Core Team, 2017) using the packages dplyr

252   (Wickham et al., 2018) and devtools (R Core Team, 2017).

253   **Results**

254   **Experiment 1: Ant mortality in response to exposure to non-entomopathogenic bacteria**

255   The effects of exposure for all ants to *S. epidermidis* and *E. coli* after 24 and at 48 h are

256   shown in Figure 1. In control groups across our experiment, mortality was low with average

257   proportions of 0.027 at 24 h and 0.064 at 48 h. In addition, across all treatments and all species

258   tested, agar discs had visible evidence of excavation upon first inspection when the 24 h

259   mortality count was made.

260   *Solenopsis invicta* showed significant mortality to exposure (Table 1; Figure 1). At 24 h,

261   there was significant mortality from *S. epidermidis* and *E. coli* (Friedman's ANOVA $F_{(2)} = 8.93$,

262   $p = 0.011$; post-hoc analyses Finner $p < 0.01$, Cohen's *d* effect sizes ≥ 0.68). At 48 h, there was

263   only significance from exposure to *E. coli* (Friedman's ANOVA 48 h $F_{(2)} = 16.3$, $p = 0.022$;

264   post-hoc analyses Finner $p = 0.003$, Cohen's *d* effect size 0.8). *Dorymyrmex bureni* also

265   displayed significant mortality to *E. coli* exposure at 24 h (Friedman's ANOVA 24 h F(2) = 23.3,

266   p < 0.0001; Finner p < 0.001, Cohen's *d* effect size 1.27) and 48 h (Friedman's ANOVA 48 h

267   F(2) = 19.7, p < 0.0001; Finner p < 0.001, Cohen's *d* effect size 5.74; Figure 1). A significant

268   treatment effect was detected for *A. rudis* at both 24 and 48 h, but in post-hoc comparison the

269   only significant difference between groups was comparing mortality bacterial treatments at 48 h

270   (Friedman's ANOVA 48 h F(2) = 10.3, p < 0.01; Finner p = 0.01, Cohen's *d* effect size 1.11).

271   All other comparisons were not significant, and mortality from both bacterial treatments was not

272   different from the control. *Brachyponera chinensis* showed no significant mortality to either

273   treatment (Table 1).

274   **Experiment 2: Does exposure to bacteria lead to an increased presence of surface**

275   **antimicrobials?**

276   Exposure seemed to have no effect on surface antimicrobial ability for both *S. invicta* and

277   *B. chinensis.* Antimicrobial ability was calculated from OD_{600} readings after WST-8 was added

278   to wells and plates were incubated for one hour. Figure 2 shows *S. invicta* has similar

279   antimicrobial ability to inhibit *E. coli* (Figure 2a) or *S. epidermidis* (Figure 2b) with and without

280   treatment exposures, *E. coli* (KW chi-squared = 0.36, df = 1, p = 0.55, epsilon square effect size

281   0.01), *S. epidermidis* (KW chi-squared = 1.3011, df = 1, p = 0.254, epsilon square effect size

282   0.045). Figure 3a shows *B. chinensis* lacks ability to completely inhibit *E. coli* but has

283   antimicrobial ability to inhibit *S. epidermidis* (Figure 3b). Exposure to bacteria did not lead to

284   significant changes in antimicrobial ability (*S. epidermidis* KW chi-squared = 0.01, df = 1, p =

285   0.92, epsilon square effect size 0; *E. coli* KW chi-squared = 2.42, df = 1, p = 0.12, epsilon square

286   effect size 0.08).

287

Discussion

288

289 Our first experiment demonstrated that exposure to a common bacterium—*E. coli*—could kill

290 two of the four ant species we tested, *S. invicta* and *D. bureni*—two species that displayed strong

291 antimicrobial ability (Penick et al., 2018). Exposure to *S. epidermidis* had no significant effect on

292 any of the tested ants except for *S. invicta* workers at 24 h of exposure. The other two ants we

293 tested, *B. chinensis* and *A. rudis* group, did not previously demonstrate antimicrobial ability nor

294 did they die from exposure to either bacterium used in this study. Our finding indicates that, like

295 antimicrobial ability, susceptibility to bacterial exposure varies across species. Additionally, our

296 results could be an indication of a linkage between an ant species' susceptibility to bacterial

297 exposure and antimicrobial ability. However, this requires further testing and considerations that

298 our experiments did not account for, as will be discussed below.

299 In our second experiment, *S. invicta* and *B. chinensis* were subjected to a sub-lethal

300 period of bacterial exposure followed by an antimicrobial assay of their surface extracts. We

301 aimed to test for a possible increase in antimicrobial ability in response to exposure to the focal

302 bacteria species. We predicted that exposure to a bacterial challenge would result in an increase

303 of antimicrobial ability from ant surface extracts, which would indicate that surface

304 antimicrobials of some ants are able to be conditionally deployed to meet direct microbial

305 challenges. As tested, we found no evidence of a change in antimicrobial ability akin to any

306 response to bacterial exposure. Fire ant, *S. invicta*, extracts demonstrated near-complete

307 inhibitory ability, as was previously found (Penick et al., 2018), against *S. epidermidis*

308 independent of previous exposure to the bacteria species. In addition, *E.coli* growth was inhibited

309 by the surface extracts of fire ants. Surface extracts from *B. chinensis* also inhibited growth of *S.*

310 *epidermidis*. This differs from the result of Penick et al. (2018) which found no effect of surface

311   extracts of *B. chinensis* on *S. epidermidis*. Our study used isopropanol as a solvent for extraction.

312   In contrast, Penick et al. (2018) used ethanol. Surface antimicrobials on *B. chinensis* may be non-

313   polar antimicrobial compounds that ethanol could not extract.

314   Our experiments focused on how ants respond to exposure to bacterial species that are not

315   known to have an evolved relationship with the ants. Our aim was to broaden our understanding

316   of how microbes might be influencing antimicrobial traits of ants. Many studies of bacteria in

317   ants focus on bacterial species that have specific, and often co-evolutionary, associations with

318   insects. For instance, intracellular bacteria that have evolved to be largely maternally transmitted.

319   *Wolbachia* species are commonly associated with insects, including ants (Ramalho, Bueno &

320   Moreau, 2017). The bacteria likely target reproductive function but do not seem to kill infected

321   adult ants (Wenseleers et al., 1998; Kautz, Rubin & Moreau, 2013). Species of the genus

322   *Spiroplasma* were found in many of the 95 ant genera considered in a recent study (Kautz, Rubin

323   & Moreau 2013). *Spiroplasma* species can be pathogens of both vertebrates and plants; Kautz,

324   Rubin & Moreau (2013) hypothesize that they might also negatively affect ants, but to date this

325   hypothesis remains untested. In another study a *Pseudomonas* species was found by Lofgren and

326   colleagues (1975) on dead ant bodies. The authors suggested that this species might have killed

327   the ants. The health effects that internal and environmental bacteria have on ants are only

328   beginning to be explored.

329   Our experiments involved exposure to extremely high concentrations of bacteria (full

330   "lawns" of bacterial growth on agar). These high concentrations are likely beyond what ants

331   normally encounter. In our experiments bacterial exposure was lethal for antimicrobial producing

332   ants, however under more natural concentrations the antimicrobial tactics the ants use might save

333   them from the mortality effects seen in our experiments. Secondly, our experiments isolated

334 workers outside of their normal nesting environments. These non-natural situations were used in

335 this first experiment as a means of assuring the ants interacted with and were exposed to the

336 bacteria. However, nesting environment and behavioral responses such as isolation and

337 avoidance of bacterial-rich areas could be integral antibacterial strategies that ants use, which

338 were not available to them in our study. Certainly, the microbiomes that ants maintain in their

339 natural nests are known to be distinct from the surrounding environment and low in number

340 potential bacterial and fungal pathogens (Lucas et al., 2019). In addition, though evidence of the

341 ants excavating the agar was observed, we did not assess the number of bacteria ingested by the

342 ants. Perhaps species that showed low mortality herein are species that ingested few or no

343 bacteria, and species that showed high mortality ingested more bacteria. Further experiments that

344 directly assess the health impacts of orally ingesting these non-ant associated bacteria would

345 likely lead to interesting insights on ant immune and antimicrobial responses.

346   Our results hint at an association between ants' use of surface antimicrobials and

347 susceptibility to bacterial exposure. We hypothesize that ants that are susceptible to a broad

348 range of bacteria are more likely to employ antimicrobials (and perhaps less likely to rely on

349 immune responses). This hypothesis could be further tested through the comparison of the

350 antimicrobial responses of a larger number of ant species, perhaps in concert with immunological

351 assays.

352   Although we were unable to demonstrate that exposure altered ants antimicrobial

353 abilities, other studies have shown that ants can immediately change their grooming rates (Reber

354 et al., 2011) and antimicrobial venom usage when threatened by pathogens (Graystock &

355 Hughes, 2011). Metapleural gland secretions in *Acromyrmex octospinosus* (Reich, 1793) leaf-

356 cutting ants were found to be significantly greater 12 h after exposure to some fungal pathogens

357   (Yek et al., 2012). It is possible that our 6 h exposure was not long enough for the ants to change

358   metapleural gland expression, or that our exposure treatments were beyond a level that would

359   induce a measurable response. In addition, our preparation of solvent extracts using isopropanol

360   may not have enabled collection of some active compounds which the ants might have been

361   producing in response to our bacterial treatments.

362   **Conclusions**

363   Ants, like humans, have evolved in the context of diverse bacterial communities for

364   millions of years. However, unlike humans, "ants" represent >16,000 species each with unique

365   life-histories, adaptations, and potential antimicrobial strategies. Like other recent comparative

366   studies in ants (e.g. Bos et al., 2019; Penick et al., 2018), our results show that responses to

367   potentially harmful microbes are varied among ant species. To ants that are susceptible to

368   microbial exposure, threats are not limited to entomopathogenic bacteria. Previous

369   pharmacological studies have demonstrated that some ants produce compounds that inhibit non-

370   ant-associated bacteria (e.g. Carvalho et al., 2019; Jouvenaz, Blum & MacConnell, 1972). These

371   studies may have been revealing that those ants are susceptible to and thus are defending against

372   exposure environmental bacteria. Further exploration of a wide range of both bacteria and ant

373   species is likely to reveal unique and nuanced antimicrobial strategies. Continued research is

374   likely to deepen our understanding of how ant societies maintain health and how we might adopt

375   some of these non-human-society strategies to better improve our own responses to microbial

376   health threats.

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384

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Figure 1

Mortality is shown for data collected at 24 h (A) and 48 h (B) when ants were exposed to *S. epidermidis* (S) or *E. coli* (E) after adjusting treatment values to control value.

15 colonies were tested for each species. Asterisks indicate statistically significant difference in mortality, relative to the control according to Friedman's rank sum test and Finner post-hoc test. Box plot line represents median values, whiskers represent 1st and 3rd quartile, data points represent ant colonies, outliers are data outside of 1.5 IQR.

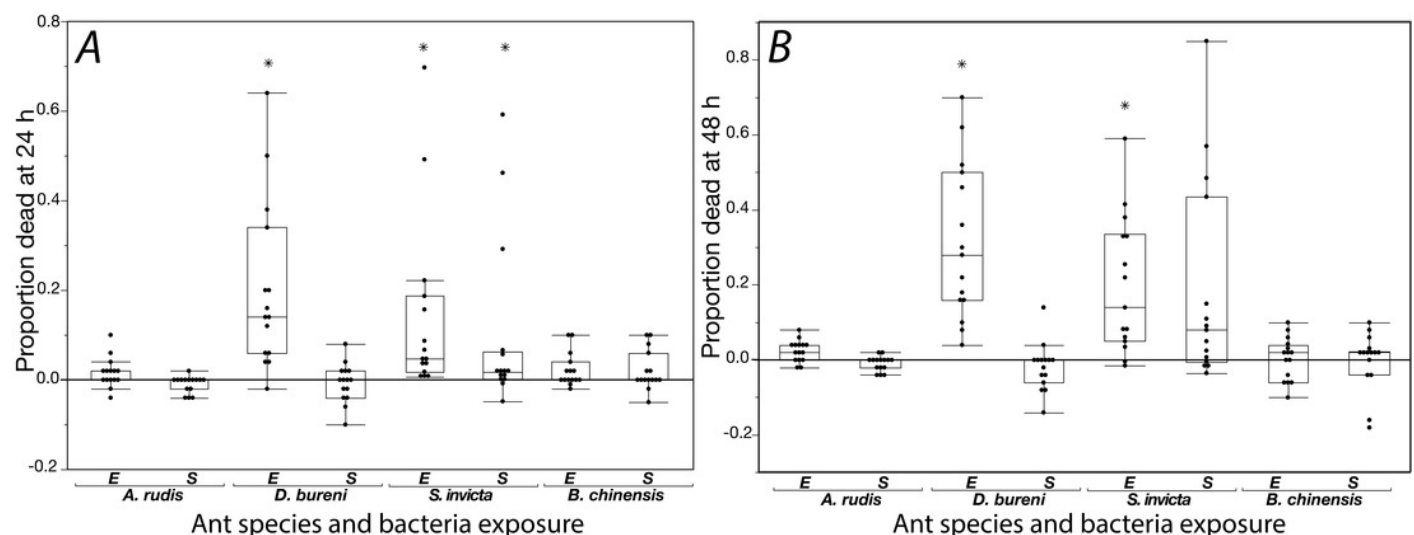



Figure 2

Extracts from *S. invicta* effectively inhibited bacterial challenge similarly with or without treatment exposures.

Bacterial inhibition of isopropanol extracts from ants when exposed to either *E. coli* (A) or *S. epidermidis* (B) or control agar (A & B). Values are percent inhibition of extracts compared to growth controls. There are no statistically significant differences between control and treatment groups. Box plot line represents median values, whiskers represent 1st and 3rd quartile, data points represent ant colonies, outliers are data outside of 1.5 IQR.

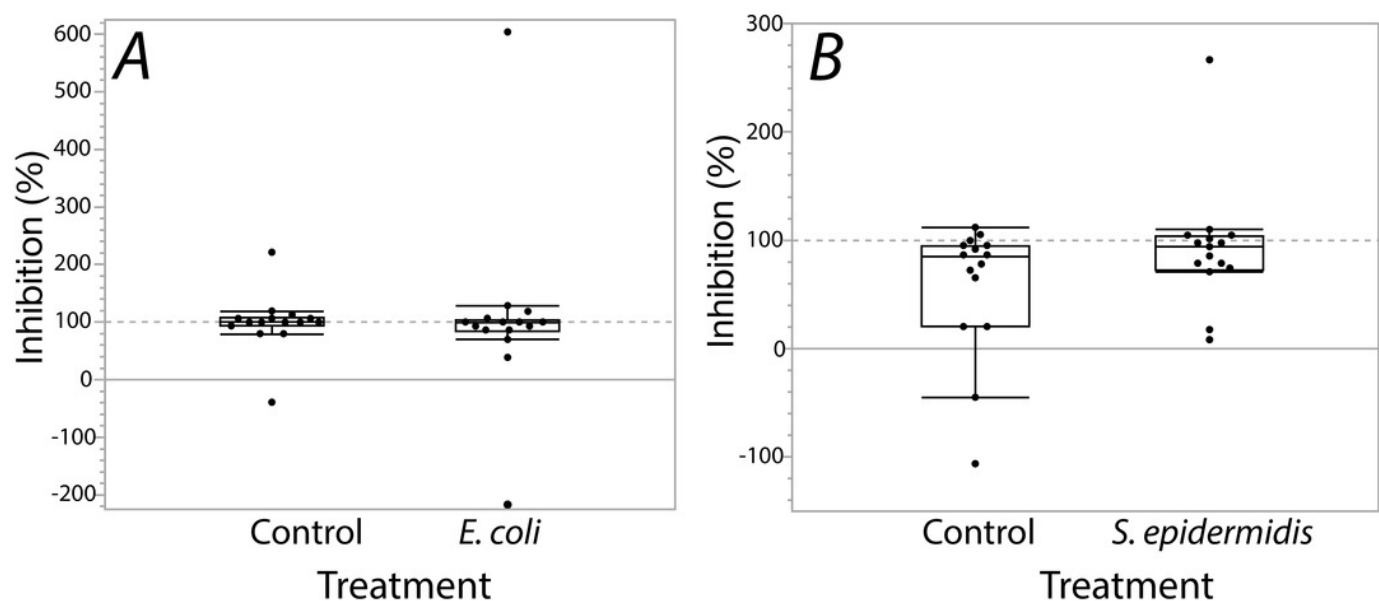
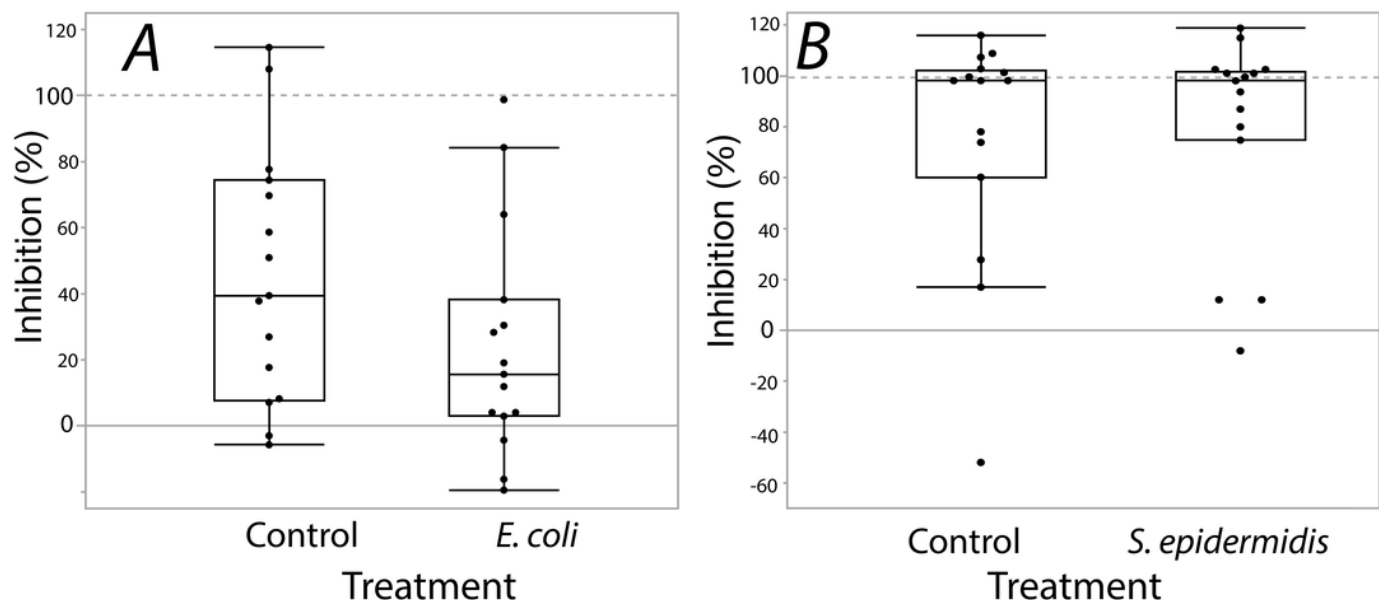



Figure 3

Extracts from *B. chinensis* showed similar bacterial inhibition ability with or without treatment exposures.

B. chinensis inhibition potential for control and treatment exposures for isopropanol extracts when exposed to *E. coli* (A) or *S. epidermidis* (B). Values are percent inhibition of extracts compared to growth controls. There are no statistically significant differences between control and treatment groups. Maximum and minimum growth controls visualized at 100% and 0%, respectfully.

Statistical comparisons for experiment 1, corresponding with Figure 1.

Bold entries correspond with statistical significance in post-hoc analyses.

1 **Table 1: Statistical comparisons for experiment 1, corresponding with Figure 1.** Bold entries correspond with statistical
2 significance in post-hoc analyses.

Species				Post-hoc comparisons: Finner p value; Cohen's *d* effect size		
24 h exposure	Friedman's chi-squared	df	p value	*E. coli* vs. control	*S. epidermidis* vs. control	*S. epidermidis* vs. *E. coli*
S. invicta	8.93	2	p = 0.011	**0.0001; 0.74**	**0.0026; 0.68**	0.41; 0.18
D. bureni	23.33	2	p < 0.0001	**0.0005; 1.27**	0.78; 0.16	**0.0003; 1.32**
A. rudis	6.63	2	p = 0.036	0.23; 0.56	0.37; 0.53	0.062; 0.92
B. chinensis	2.23	2	p = 0.33			
48 h exposure						
S. invicta	16.3	2	p = 0.022	**0.021; 0.8**	0.087; 0.69	0.42; 0.04
D. bureni	19.733	2	p < 0.0001	**0.0005; 5.74**	0.42; 0.34	**0.00003; 5.95**
A. rudis	10.03	2	p = 0.0066	0.07; 0.77	0.37; 0.38	**0.011; 1.11**
B. chinensis	2.84	2	p = 0.24			