Experimental modulation of external microbiome affects nestmate recognition in harvester ants (*Pogonomyrmex barbatus*)

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Social insects use odors as cues for a variety of behavioral responses, including nestmate recognition. Past research on nestmate recognition indicates cuticular hydrocarbons are important nestmate discriminators for social insects, but other factors are likely to contribute to colony-specific odors. Here we experimentally tested whether external microbes contribute to nestmate recognition in red harvester ants (*Pogonomyrmex barbatus*). We changed the external microbiome of ants through topical application of either antibiotics or microbial cultures. We then observed behavior of nestmates when treated ants were returned to the nest. Ants whose external microbiome was augmented with microbial cultures were much more likely to be rejected than controls, but ants treated with antibiotics were not. This result is consistent with the possibility that external microbes are used for nestmate recognition.

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2   harvester ants (*Pogonomyrmex barbatus*)

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20   ABSTRACT

21   Social insects use odors as cues for a variety of behavioral responses, including nestmate

22   recognition. Past research on nestmate recognition indicates cuticular hydrocarbons are important

23   nestmate discriminators for social insects, but other factors are likely to contribute to colony-

24   specific odors. Here we experimentally tested whether external microbes contribute to nestmate

25   recognition in red harvester ants (*Pogonomyrmex barbatus*). We changed the external

26   microbiome of ants through topical application of either antibiotics or microbial cultures. We then

27   observed behavior of nestmates when treated ants were returned to the nest. Ants whose external

28   microbiome was augmented with microbial cultures were much more likely to be rejected than

29   controls, but ants treated with antibiotics were not. This result is consistent with the possibility

30   that external microbes are used for nestmate recognition.

31   *Keywords: Nestmate recognition, microbiome, harvester ants*

32   INTRODUCTION

33   In ants, individuals discriminate between nestmates and non-nestmates, which is often equivalent

34   to kin discrimination, by using chemical cues (Vander Meer and Morel 1998; van Zweden and

35   d'Ettorre 2010; Sturgis and Gordon 2012). Cuticular hydrocarbons (CHCs) are chemical

36   compounds that serve a variety of functions in social insects but very often are implicated in

37   nestmate recognition by both empirical and theoretical research (Wagner et al. 2000, Howard and

38   Blomquist 2005). CHCs are synthesized from lipids, and the CHC profile of an ant, which

39   consists of various classes of alkanes, alkenes and methyl-branched hydrocarbons in differing

40   proportions, can be both task and colony specific (Sturgis and Gordon 2012). In *Cataglyphis*

41   *niger*, when hydrocarbons are isolated from the postpharyngeal gland and applied to one of a pair

42   of live ants, the untreated ant behaves in a way that indicates CHCs function as nestmate

43   discriminators (Lahav et al. 1999). Esponda and Gordon (2015) recently proposed a model of

44   distributed nestmate recognition in which each ant in a colony has a flexible decision boundary in

45   multidimensional odor space, acquired through interactions, that divides nestmates from non-

46   nestmates.

47   For good reason, research on nestmate recognition in social insects has focused on CHCs, but

48   other chemicals are also likely to play a role in nestmate recognition (Sturgis and Gordon 2012).

49   Davis et al. (2013) reviewed studies showing that volatile chemicals emitted by microbes affect

50   insect behavior ranging from foraging to oviposition. Lizé et al. (2014) showed that bacteria

51   influence kin recognition in *Drosophila melanogaster*. Although CHCs alone are sufficient for

52   nestmate recognition in some species of ants (Lahav et al. 1999; Wagner et al. 2000), this does

53   not preclude a role for microbes. Both internal and external microbial communities are colony-

54   specific in ants (Anderson et al. 2013; Hu et al. 2014), and thus potential nestmate discriminators.

55   Over ten years ago, Matsuura (2001) experimentally showed that bacteria mediate nestmate

56   recognition in termites (*Reticulitermes speratus*). However, the role of microbes in nestmate

57   recognition of ants has not been investigated, except for a study by Richard et al. (2007) showing

58   that ants from one colony of fungus growing ants (*Acromyrmex spp.*), if reared on a second

59   colony's fungus, experienced less aggression when introduced to the second colony.

60   Here we test the hypothesis that the microbial composition on an ant's cuticle affects whether it is

61   treated as a nestmate. Both pathogenic and protective microbes inhabit the surface of insect

62   cuticles (Douglas 2015), and they could produce chemicals that affect nestmate recognition

63   (Davis et al. 2013). Previous work in honey bees (*Apis mellifera*) showed that internal injection

64   of bacteria caused significant changes in CHC profiles, and in turn nestmate recognition (Richard

65   et al. 2012), but here we isolate the effect of external microbes on nestmate recognition. To test

66   the hypothesis that the external microbiome affects nestmate recognition in ants, we

67   experimentally modulated the microbes on the cuticles of red harvester ants (*Pogonomyrmex*

68   *barbatus*) and tested whether those ants were rejected by their nestmates. *P. barbatus* can

69   distinguish between nestmates and non-nestmates using CHCs alone (Wagner et al. 2000), but

70   microbes may still play a role. We predicted that if the external microbiome plays a role in

71   nestmate recognition, individuals with an altered external microbiome would not be accepted as

72   nestmates when returned to their nest.

73   METHODS

74   *Study species*

75   Experiments were conducted in 3 queenright, brood-producing laboratory colonies of red

76   harvester ants (*Pogonomyrmex barbatus*) obtained from a field site near Rodeo, NM. Details of

77   lab conditions are found in Gordon et al. (2005).

78   *Assay of nestmate recognition*

79   On the day of the experiment, we first removed a group of ants from their nest's foraging arena

80   ~4 hours prior to behavioral testing. Group size (9-20 ants) depended on the number of ants

81   active in the foraging arena during sampling. Each ant then received one of five experimental or

82   control treatments, described below, and was painted with a color corresponding to its treatment

83   for identification during behavioral observation (Gordon et al. 2005). We then returned the group

84   of treated and marked ants to a location near the nest entrance, where they interacted with

85   resident nestmates. We performed the test with groups of ants at the nest entrance, rather than

86   paired individuals in a foreign arena, because recent models indicate that accurate nestmate

87   recognition occurs at the colony level (Johnson et al. 2011; Esponda and Gordon 2015) and

88   empirical work indicates that testing ants in groups provides results that are more consistent and

89   indicative of natural responses than tests with individuals (Roulston et al. 2003). An observer

90   blind to the treatment groups then scored whether each ant was rejected from the nest during the

91   following 20 minutes. Rejection, which was often preceded by aggressive acts like biting,

92   consisted of a resident ant seizing a treated ant and carrying it out of the nest or away from the

93   nest entrance towards the midden pile at the far end of the foraging arena.

94   *Experimental treatment*

95   We manipulated the external microbiome of ants (N = 111) using five different treatments: 1)

96   Antibiotic group: To decrease the external microbiome, we coated each ant's body surface with a

97   1% rifampin/sterile water solution. 2) Microbial group: To increase the external microbiome, we

98   coated each ant's body surface with a solution of Lysogeny Broth (LB) broth (Becton Dickenson,

99   Franklin Lakes, NJ) that had previously been cultured from nestmates. Because only a small

100   proportion of the microbiome survives in culture (Rappe and Giovannoni 2003), this solution

101   modified and augmented an ant's external microbiome, and we then confirmed this change (see

102   below). In the control groups, we treated ants with 3) Water: sterile water, 4) Broth: sterile LB

103   broth, or 5) Nest: simply removed ants from the nest and marked them before return.

Validation of treatment

105   To validate that our treatment produced the intended effect on the ants' external microbiome, we

106   used the method of Ren et al. (2007) to culture the external microbiome of additional ants (N =

107   35). Although only a small proportion of microbial species can be cultured (Rappe and

108   Giovannoni 2003), we used differences in the amount of culturable microbes obtained, measured

109   by counting the number of microbial colony forming units (CFUs) that grew on LB agar plates, to

110   indicate effectiveness of the treatments. A greater number of CFUs indicates a greater amount of

111   external microbes on an ant.

Effect of treatment on survival

113   To determine whether the treatments had any beneficial or adverse effects, we treated additional

114   ants (N = 72) and kept them in Petri dishes with water for two weeks. We checked on individuals

115   once daily and recorded whether the ant was alive or dead.

Statistical analysis

117   To control for non-independence produced by colony membership and returning ants in groups

118   (i.e. block design), we used a generalized linear mixed-effects model fitting block and colony as

119   random effects, with treatment (Microbial, Antibiotic, LB Broth, Water, Nest) as a categorical

120   predictor and rejection as a binary dependent variable. We used the *lmer* function in the *lme4*

121   package in R 3.0.2 for linear mixed-effects models, and the *multcomp* package to make Tukey

122   post hoc comparisons of treatment effects.

123   Because the counts of CFUs were nonparametrically distributed, we used Wilcoxon Rank Sum

124   Tests to assess the treatment validation. We made planned comparisons that would indicate

125   treatment efficacy (Antibiotic vs. Broth, Water, and Nest; Microbial vs. Broth, Water, and Nest),

126   correcting alpha for the false discovery rate according to Benjamini and Hochberg (1995).

127   To determine whether an experimental treatment significantly increased or decreased survival, we

128   used a Cox proportional hazards model with the *survival* package in R (Crawley 2007), and used

129   the *multcomp* package to make Tukey post hoc comparisons of treatment effects.

130   RESULTS

131   The experimental treatment modulated ants' external microbiome, as measured by number of

132   colony forming units. The topical application of the microbial culture significantly increased in

133   the number of CFUs obtained from an ant's cuticle compared to each of the controls (Microbial

134   vs. Broth: $W = 49$, $P = 0.002$, $N = 10$, vs. Water: $W = 40.5$, $P = 0.006$, $N = 9$, vs. Nest: $W = 49$, P

135    $= 0.002$, $N = 10$). Antibiotic treatment significantly decreased the number of CFUs compared to

136   controls, except for the water treatment (Antibiotic vs. Broth: $W = 7.5$, $P = 0.035$, $N = 10$; vs.

137   Water: $W = 13.5$, $P = 0.317$, $N = 9$; vs. Nest: $W = 7$, $P = 0.026$, $N = 10$). With 5 of 6 comparisons

138    $P < 0.05$, our alpha, controlled for false discovery rate, is 0.042.

139   Changes in an ant's external microbiome influenced whether it was rejected from its nest by

140   nestmates (Figure 1). Ants with experimentally augmented external microbiomes were more

141   likely to be rejected than ants treated with broth alone, sterile water, or only marked (all post hoc

142   comparisons $|Z| > 3.8$, $P < 0.001$). Ants treated with the antibiotic solution were not more likely

143   to be rejected than ants treated with broth alone, sterile water, or only marked (all post hoc

144   comparisons $|Z| < 1.1$, $P > 0.80$).

145   Ants in the Microbial group died significantly sooner than ants given the water treatment ($Z =$

146   -3.085, $P = 0.017$). There were no significant differences in survival between the Microbial group

147   and the other control groups (all post hoc comparisons $|Z| < 1.64$, $P > 0.46$), or between the

148   Antibiotic group and the control groups (all post hoc comparisons $|Z| < 1.173$, $P > 0.76$).

149   DISCUSSION

150   We found that as we predicted, ants treated with additional microbes were rejected by resident

151   colony members as if they were non-nestmates. This aligns with our predictions and supports our

152   hypothesis that the external microbiome plays a role in nestmate recognition in *P. barbatus*.

153   Contrary to predictions, ants treated with the antibiotic solution were not rejected by resident

154   colony members. Because the antibiotic treatment was delivered in a sterile water solution, ants'

155   CHC profiles were likely unaltered, in which case many familiar odors would persist. This is

156   consistent with the possibility that the presence of a foreign odor is more likely to lead to the

157   recognition of a non-nestmate than absence of a familiar odor (van Zweden and d'Ettorre 2010;

158   Esponda and Gordon 2015), as well as experimental data showing that carpenter ants

159   (*Camponotus herculeanus*) reject non-nestmates by recognizing novel CHCs (Guerrieri et al.

160   2009).

161   The rejection of ants with augmented external microbiomes could be interpreted as a form of

162   social immunity (Cremer et al. 2007), in which ants with an unfamiliar or unusual microbiome

163   are perceived as sick nestmates in need of quarantine. Social immunity may involve a

164   microbially-mediated form of nestmate recognition, since a microbe that causes an individual to

165   be placed on the 'reject' side of another ant's boundary may or may not be a pathogen. In this

166   way, social immunity could constitute an additional function of microbially-mediated nestmate

167   recognition. We found a significant difference in survival between ants from the Microbial group

168   and those treated with water, but not between ants from the Microbial group and those from the

169   Broth and Nest groups. Thus, the extent to which the microbial treatment may have included

170   pathogens is not resolved and we do not know the extent to which rejection of treated ants

171   contributed to social immunity.

172   In addition to further research on social immunity, our results suggest several research directions

173   that may provide insight on the role of microbes in chemical communication. First, it will be

174   important to learn what microbial species are key to nestmate recognition and what chemicals

175   they produce. In hyenas, fermentative bacteria appear to play a key role in chemical

176   communication (Theis et al. 2013). Second, it will be important to establish how much microbial

177   communities differ among colonies, and how consistent those communities are over time. In

178   leafcutter ants (*Acromyrmex echinatior*), the external microbiome is colony-specific and

179   differences are maintained up to 10 years, even after colonies were kept in laboratory conditions

180   (Anderson et al. 2013). Third, an ant's microbiome may be task specific, as well as colony-

181   specific. In *P. barbatus*, CHCs are task specific (Sturgis and Gordon 2013) and influence task

182   allocation (Greene and Gordon 2003). It is likely that microbial communities of ants vary with

183   task type, as foragers are exposed to different microbes from brood care or midden workers

184 (Cremer et al. 2007). Microbes may also inform task decisions alongside CHCs, or could directly

185 affect the structure of CHCs.

186 In conclusion, our results support a role for microbes in nestmate recognition in harvester ants.

187 They align with experimental data showing microbes influence nestmate/non-nestmate

188 recognition in termites (Matsuura 2001) and leaf-cutting ants (Richard et al. 2007), and show that

189 external microbes alone can impact nestmate recognition. This finding augments extensive

190 evidence of CHC-mediated nestmate recognition in social insects (Vander Meer and Morel 1998;

191 van Zweden and d'Ettorre 2010; Sturgis and Gordon 2012), and calls for further investigation of

192 the role of microbes in chemical communication of social insects.

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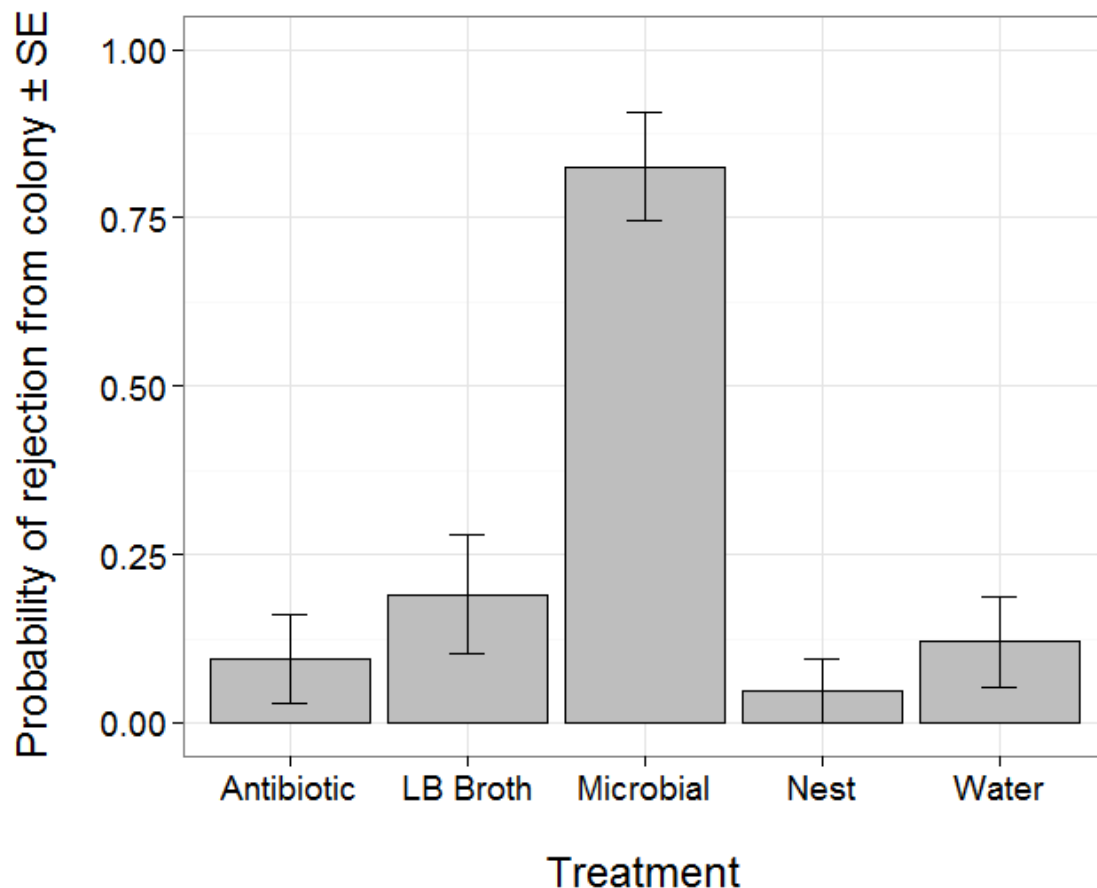
248   FIGURE

249   Figure 1 – Probability that an ant was rejected by its nestmates (i.e. not recognized as a nestmate)

250   after manipulation of its external microbiome. Antibiotic = topical treatment with 1% rifampin

251   solution. LB Broth = topical treatment with sterile LB broth. Microbial = topical treatment with

252   microbes cultured from ants in LB broth. Nest = no topical treatment; individuals were only

253   marked before return to nest. Water = topical treatment with sterile water.