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**Poor Odors, Strength, and Persistence Give Their Rewards to *Mutilla europaea* Visiting Dangerous Wasp Nests**

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12 **Poor odours, strength, and persistence give their rewards: the strategy of**

13 ***Mutilla europaea.***

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27        **ABSTRACT**

28        Social insect colonies are attractive for many arthropods. The rare velvet ant, *Mutilla europaea*,  
29 visit colonies of *Polistes* wasps, but to date it was unclear which resources it targeted therein. Our  
30 field observations and bioassays showed that velvet ants visit the colonies of the social wasp  
31 *Polistes biglumis* almost undisturbed and probably feed on larval wasp saliva. Chemical  
32 insignificance and resistance are the characteristics that allow velvet ants to visit unharmed wasp  
33 colonies and gain such a nutritious reward.

34

35        Key words: cleptoparasitism, *M. europaea*, *P. biglumis*, social insects, larval wasp saliva

36

37

38           **INTRODUCTION**

39           Social insect colonies are an incredibly valuable source of resources. They are composed by  
40 numerous, related individuals that endoparasites and pathogens can use as hosts and a worker caste  
41 that social parasites can exploit; they contain food storage and immature brood that other animals  
42 can prey upon and nests that they can use as shelters. It is not surprising, then, that social insect  
43 colonies are infested by arthropods which penetrate, depredate or exploit the colony resources at  
44 various extent (cleptoparasites, Evans and West-Eberhard 1970; for general reviews, Schmid-  
45 Hempel 1998; social predators, Nash and Boomsma 2008). Among other parasites, mutillid wasps  
46 visit social insect colonies (Brothers et al. 2000 and references therein). For example, the females of  
47 the velvet ant *Mutilla europaea* Linnaeus (Hymenoptera: Mutillidae) enter bumblebee colonies to  
48 reproduce as bumblebee parasitoids. Moreover, they have also been observed visiting *Polistes spp.*  
49 colonies. Although velvet ants possibly search wasp colonies for food (Brothers et al. 2000), what  
50 resource they target therein is unknown. *M. europaea* has a poorly known natural history because it  
51 is extremely rare (Brothers et al. 2000). Adults are sexually dimorphic; males are smaller than  
52 females and have wings, whereas females are wingless, brilliantly coloured and have a hardened  
53 cuticle (Evans and West-Eberhard 1970).

54           During our long-term study on populations of *Polistes biglumis* Linnaeus (Hymenoptera:  
55 Vespidae), we have noticed several times that females of *M. europaea* visit these wasps' colonies  
56 (M.C.L. pers. obs.). Wasps from the family Vespidae are known for having very good recognition  
57 abilities (Lorenzi et al. 1997) and therefore it is reasonable to ask how female velvet ants can visit  
58 their colonies and how the residents respond to these intruders. We performed behavioural  
59 observations in field *P. biglumis* colonies to detect which resources velvet ants target and  
60 recognition bioassays to identify how resident wasps defend their colonies against velvet ants.

61

62           **MATERIALS AND METHODS**

63        **Study sites**

64        The study was performed over two summers in 2006 and 2007, at two different sites in the  
65        Western Alps (Montgenèvre, Hautes Alpes, France and Ferrere, Val Stura di Demonte, Cuneo,  
66        Italy), where two dense *P. biglumis* populations live.

67

68        **Behavioural observations of naturally occurring visits of wasp nests by velvet ant females**

69        A total of 117 *P. biglumis* colonies were located: 78 were in the pre-emergence period (i.e.,  
70        founding phase, when the colony is composed of its single foundress and her immature brood) and  
71        39 in the post-emergence period (i.e., the colony is composed of its single foundress and her adult  
72        and immature brood). We carried out 222 hours of field colony observation (about 2h per colony).  
73        We recorded the content of each nest cell (i.e. whether the cell was empty or contained eggs, larvae,  
74        pupae or honey) before and after each observation session.

75        During the field colony observations, only 3 velvet ants visited each a wasp colony. In the  
76        analysis of these observational data, we focused on the velvet ants and calculated the number of  
77        bites they received by the resident wasp(s), the time velvet ants were under attack, the time they  
78        spent walking on the nest or headfirst in each nest cell (the only behaviours velvet ants exhibited  
79        while on wasp nests). Additional behavioural data on velvet ants visiting wasp nests were collected  
80        by A.U. who observed 2 velvet ants females each visiting a wasp nest and noted their behaviour and  
81        the nest-cell contents. Thus, data on the behaviour of velvet ants on wasp nests come from 5  
82        individuals.

83

84        **Sample collection for recognition bioassays**

85        We could not collect large numbers of samples because velvet ants are rare and difficult to  
86        collect. We rarely saw them in field (about 20 specimens were seen over the two seasons of study).  
87        Furthermore, velvet ant females are fast, armed with a sting, and difficult to capture. However, we

88 were able to collect 9 velvet ant females for behavioural tests by direct sightings and dry pitfall  
89 traps. Pitfall traps were largely unsuccessful (we trapped 3 females in 153 traps placed).

90 We also collected 3 *P. biglumis* foundresses from their pre-emergence colonies.

91 Wasps and velvet ants were freeze-killed and stored in freezer until they were used as dead  
92 insects in recognition bioassays.

93

#### 94 **Recognition bioassays**

95 Interactions between velvet ant and *P. biglumis* occurred rarely in the field. Therefore, we  
96 simulated the visit of wasp nests by velvet ants and tested the response of *P. biglumis* wasps to  
97 velvet ant by performing recognition bioassays in the field. Several studies have used recognition  
98 bioassays in the past to simulate visits of insects on wasp nests (e.g., Bonavita-Cougourdan et al.  
99 1991; Lorenzi et al. 1997; Ruther et al. 2002; Cristina Lorenzi et al. 2011).

100 We performed the bioassays on a total of 90 *P. biglumis* colonies in the two study sites. Of the  
101 90 colonies, 71 were in the pre- and 19 in the post-emergence period. We artificially introduced into  
102 the 71 pre-emergence colonies each of the following: 1) a dead velvet ant female (n = 6); 2) a dead  
103 velvet ant female that had previously been washed in 1 ml pentane for 1 h to remove the  
104 epicuticular lipids (n = 3); and 3) a *P. biglumis* foundress (i.e. foundresses of alien colonies,  
105 potential usurpers) (n = 3). For logistic reasons (e.g., change of weather, wasp(s) leaving the nest),  
106 we could not perform the three tests in all the nests; in the results, the largest sample size for each  
107 comparison is reported. Additionally, we tested the reaction of post-emergence colonies to velvet  
108 ants by introducing one of two dead velvet ant females into the 19 post-emergence colonies.

109 The bioassays were performed in undisturbed, non-manipulated wasp colonies during sunny  
110 days, within the activity period of the wasps, by presenting colonies with one dead insect at a time.  
111 Dead insects were presented in random order, at intervals > 10 min. We checked for potential  
112 effects of the rank of presentation of *Mutilla* females on wasps' responses by means of  $r_s$ . Dead

113 insects were held with forceps, placed 1 cm from the anterior face of the nest, and kept there for 1  
114 min from the first unambiguous reactions by the resident wasp(s). We counted how many attacks  
115 (bites, grasping, darts; see Table 1 for a description) resident wasp(s) exhibited towards the  
116 presented insect. In pre-emergence colonies the number of foundresses was always 1. In post-  
117 emergence colonies, we divided the total number of attacks by the number of resident wasps which  
118 were on the anterior face of the nest during the bioassay. In this way, each tested colony supplied a  
119 single datum. Subsequently, we compared the number of attacks and the number of grasps (the most  
120 aggressive behaviour) that each dead insect received in each test, using the Wilcoxon test. We  
121 tested whether wasp responses were affected by the rank of presentation of dead insects using  
122 Spearman  $r_s$  test.

123 Tests were not blind because of obvious morphological differences between species. However,  
124 reactions by resident wasps were sufficiently clearly cut to avoid subjective interpretation.

125 Statistical analyses were carried out using the statistical program SPSS 17.0.

126

## 127        **RESULTS**

### 128        **Behavioural observations of naturally occurring nest visits by velvet ant females**

129        During the 222 hours of field observations of *P. biglumis* colonies, we recorded only 3 visits by  
130 velvet ant females, of which 1 occurred in a pre-emergence colony and 2 in post-emergence  
131 colonies. Visits lasted respectively 31, 90 and 27 min.

132        In the three visits, velvet ants were rarely attacked by resident wasps. Indeed, they were under  
133 attack on average for only 3.79% of the time they spent on the wasp nests and were ignored by the  
134 wasps for the remaining time. Attacks were more frequent in the first 10 min of each visit than later  
135 (although the difference is not significant: Wilcoxon test,  $N = 3$ ,  $Z = -1.604$ ,  $P = 0.109$ ). The two  
136 additional visits by velvet ants which we happened to see occurred in pre-emergence colonies and  
137 lasted 6 and 15 min respectively (from the moment we started the observation).

138        Overall, the 5 velvet ant females spent on average 69.87% of the time (range 50.7 - 83.3%)  
139 headfirst in the cells of *P. biglumis* nests, with only the tips of their abdomens protruding. Velvet  
140 ants never visited cells with honey stores. Instead, they spent significantly more time (up to 12 min)  
141 headfirst in cells containing *P. biglumis* larvae than in any other cells, i.e.empty, with eggs, or with  
142 pupae (Wilcoxon test,  $N = 5$ ,  $Z = -2.023$ ,  $P = 0.043$ ). Wasp larvae had no visible damages after  
143 velvet ants left the cells. Velvet ants spent the rest of the time on nests walking. They had no liquid  
144 food exchange with adult wasps.

145

### 146        **Recognition bioassays**

147        During our bioassays, wasp responses were not affected by the rank of presentation of dead  
148 insects ( $r_s$ ,  $P > 0.4$ , all comparisons).

149        When placed in front of a wasp nest (i.e. simulating a visit of the wasp colony), velvet ants  
150 were moderately attacked by resident foundresses, but attacks were significantly less than those to  
151 foundresses from alien colonies (Wilcoxon test,  $N = 41$ ,  $Z = -2.710$ ,  $P = 0.007$ ) (fig. 1). Grasps were

152 also less toward *M. europaea* females than toward alien conspecifics ( $N = 41$ ,  $Z = -4.894$ ,  $P =$   
153  $0.000$ ). Velvet ants washed in pentane were attacked significantly less than intact velvet ants ( $N =$   
154  $47$ ,  $Z = -2.074$ ,  $P = 0.038$ ).

155 Velvet ants received similar numbers of attacks in pre- and post-emergence colonies (Mann-  
156 Whitney U test,  $N_1 = 71$ ,  $N_2 = 19$ ,  $U = 642$ ,  $P = 0.747$ ).

157

## 158 **DISCUSSION**

159 Our field observations suggest that velvet ants may be cleptoparasites of the social wasp *P.*  
160 *biglumis*. During naturally occurring visits of *P. biglumis* nests, velvet ants ignored adult wasps as  
161 well as cells containing honey stores. Instead, velvet ants spent long periods of time headfirst in the  
162 cells which contained the host larvae (up to 12 min/cell). Wasp larvae had no visible damage after  
163 they were visited by velvet ants. The larvae of social wasps have huge salivary glands which open  
164 near their mouth. Larvae saliva is particularly nutritious, i.e. an important source of free amino-  
165 acids (reviewed in Hunt 2007), contains antibiotics (Turillazzi et al. 2004), and influences fecundity  
166 (Børgesen and Jensen 1995). Adult wasps often imbibe it after tactile stimulation as a food  
167 supplement (Cummings et al. 1999), performing the so-called trophallaxis (Wheeler 1918).  
168 Savoyard et al. (1998) also hypothesize that wasps perform special behaviors to communicate  
169 larvae to stop producing saliva when the nest is unattended (but see Jeanne and Suryanarayanan  
170 2011).. We hypothesize that velvet ants visit *P. biglumis* colonies to obtain saliva from the host  
171 larvae.

172

173 Unexpectedly, wasp colonies seem relatively undefended against velvet ants. During our  
174 observations, resident wasps attacked the velvet ants for less than 4 % of the time the intruder spent  
175 on the nests. During the remaining time, resident wasps were involved in their routine colony  
176 activity. When wasps patrolled their nests and occasionally met velvet ants, wasps mostly ignored

177 them. These observations suggest that velvet ants do not use propaganda or appeasement substances  
178 or repellents, since these substances should alter the behaviour of resident insects (e.g., D'Ettorre et  
179 al. 2000; Mori et al. 2000; Ruano et al. 2005). Our recognition bioassays also showed that *M.*  
180 *europaea* is usually attacked less than an alien *P. biglumis* foundress. Moreover, the reaction to a *M.*  
181 *europaea* is usually less aggressive than to a conspecific from another colony. These results also  
182 suggest that wasps estimate the potential intrusion of an alien foundress into their nests as riskier  
183 than that of a velvet ant. Indeed, conspecific foundresses cause a dramatic loss in fitness to  
184 foundresses, as they behave as social parasites in alien colonies. They displace the legitimate  
185 foundress, mark the host colony with their odour, destroy part of its brood and enslave the other to  
186 rear their own brood (Lorenzi and Cervo 1995; Lorenzi et al. in press). In comparison, velvet ants  
187 cause relatively small damages to the target colonies, as our data show that no larva had visible  
188 injuries after velvet ant visited them.

189       Chemical analyses of the epicuticular lipids have recently shown that velvet ants have less  
190 recognition cues than their *P. biglumis* hosts (Uboni et al., 2012). Therefore, chemical  
191 insignificance may allow velvet ants to visit wasp nests without being significantly attacked.  
192 Nevertheless, a few attacks happened during our field observations. These attacks were probably  
193 provoked by the few lipids that covered the velvet ant epicuticle, as demonstrated by our bioassays  
194 where washed *M. europaea* females were attacked less than intact velvet ants, but they were not  
195 enough to stop velvet ants from visiting the host colonies. During our field studies, we also  
196 observed some *M. europaea* males trying to approach *P. biglumis* nests and failing. Males and  
197 females of *M. europaea* do not differ in their chemical profile (A.U. M.C.L., and A.-G. Bagnères,  
198 unpublished). We hypothesise that the hardened cuticle of *M. europaea* females help them resist to  
199 the few attacks they receive from the resident wasps when they visit their colonies.

200       We conclude that a hard cuticle and a poor chemical profile are essential features that let a  
201 cleptoparasite enter wasp colonies almost unharmed and gain nutritious food rewards.

202

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206

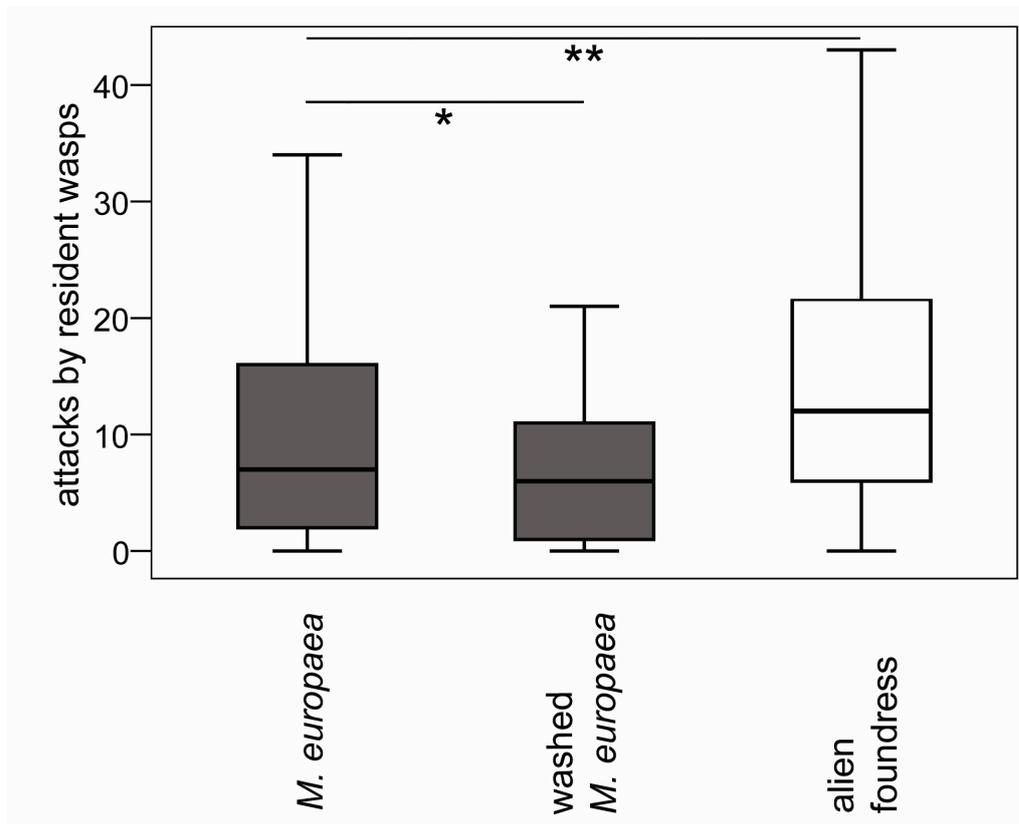
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- 251  
252

254 Table 1. Description of the aggressive behaviours recorded during our recognition bioassays.

Name	Description
Bite	A resident wasp stays on the nest with at least four legs and bites the dead insect with its mandibles.
Grasp	A resident wasp leaves the nest, flies to the dead insect, and grasps it.
Dart	A resident wasp performs a thrust against the dead insect, without touching it.

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256



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258

259 Fig 1. Results of recognition bioassays. Box plots show the number of attacks exhibited by  
 260 resident wasp(s) against velvet ant (grey), either intact or pentane-washed, and alien *P. biglumis*  
 261 foundresses (white) (\* =  $P < 0.05$ ; \*\* =  $P < 0.01$ ).