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Cryptic castes, social context and colony defence in a social bee, *Tetragonula carbonaria*

Short title: Cryptic castes and colony defence in a social bee

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Conflict of interest

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34 We declare we have no conflict of interest

35

36 **Abstract**

37 Division of labour in social insect colonies is facilitated in two ways: through temporal
38 sharing of tasks or by morphologically specialised castes. In casteless species, colony
39 defence is maintained by morphologically indistinct workers, who lack the obvious defensive
40 specialization of polymorphic species. Discrimination of intruders is carried out via antenna,
41 which also detect defensive social cues such as alarm pheromones. Despite their functional
42 importance however, antennal morphology is rarely considered in studies of nestmate
43 recognition. We investigated antennal morphology and the necessity of social cues in
44 mediating defensive behaviour across differentially tasked workers of a casteless social bee,
45 *Tetragonula carbonaria*. Our results suggest that the current understanding of division of
46 labour in casteless worker species remains poorly understood, with differences in antennal
47 morphology and aggression creating morphologically and behaviourally distinct 'cryptic
48 castes'. Further, we found that defensive behaviour was only elicited near nest odours,
49 highlighting the importance of mediating aggression among workers.

50

51 **Keywords:** caste, nestmate recognition, task allocation, antenna, *Tetragonula carbonaria*

52

53 **Introduction**

54 Division of labour in social insect societies is essential for colony organization and
55 maintenance. Often, it is facilitated by worker dimorphism and the evolution of highly
56 specialised castes (Wilson 1984; Fjerdingstad & Crozier 2006). In these societies, workers
57 are behaviourally variable and task allocation is static. Guards are among the most
58 specialised castes in highly polymorphic species, possessing distinctive morphological and
59 behavioural characteristics: nestmate recognition studies repeatedly reveal that these
60 workers are more aggressive than their typically smaller nestmates (Whitehouse & Jaffe
61 1996; Grüter et al. 2012). In contrast, task allocation changes temporally in species with
62 morphologically indistinct workers. Typically, workers in these species spend the first weeks
63 after eclosion engaging in in-nest duties such as nursing, then progress to guard behaviour
64 and eventually foraging (Robinson 1987; Gordon et al. 2005). In these casteless worker
65 societies, a lack of specialization suggests that each individual is similarly capable of
66 engaging with intruders and defending the nest.

67

68 Nest defence is of paramount importance to social insects that are frequently attacked by
69 predators, parasites and conspecific intruders (Breed & Page 1991). Nest defence in both

70 casteless and polymorphic worker species requires two steps: distinguishing between
71 nestmates and others, and an appropriate behavioural response (Leonhardt et al. 2016).
72 Among bees, sensilla on the antennae and legs detect the cuticular profiles (CHC's) of other
73 individuals. Olfactory receptors in the sensilla bind odour molecules, stimulating sensory
74 neurons that are then decoded in specialised regions of the brain (Kaissling 1971;
75 Hildebrand 1995). Bees use CHC's to distinguish between nestmates and other intruders,
76 and an unfamiliar profile typically causes an aggressive response (van Zweden & d'Ettorre
77 2010).

78

79 Antennae have an additional function involved with nest defence – the detection of cues
80 that may motivate workers to react aggressively. Volatile signals, such as alarm
81 pheromones, encourage defensive behaviour (Blum 1969), but more subtle social signals
82 emanating from the nest may similarly activate a response (Couvillon et al. 2013). Given the
83 functional importance of antennae, it is surprising that antennal morphology is rarely, if ever,
84 considered in studies of nestmate recognition (but see (Gill et al. 2013)).

85

86 Workers in the eusocial meliponine bee *Tetragonula carbonaria* are morphologically
87 identical and, like other meliponine species (Sakagami 1982; Sommeijer 1984; Inoue et al.
88 1996; Grüter et al. 2017), task allocation is assumed to be associated with age, although
89 some meliponine species have evolved behaviourally distinct sub-castes (Hammel et al.
90 2016; Grüter et al. 2017). Guards in *T. carbonaria* are visible at the entrance and challenge
91 returning workers and potential intruders (Gloag et al. 2008). If an intruder is detected, they
92 are removed by a guard in a physical contest that is usually fatal for both combatants,
93 ensuring the cost of misidentification is high (Gloag et al. 2008; Cunningham et al. 2014;
94 Shackleton et al. 2015). We examined variation in antennal morphology and social cues
95 across guards and foragers in this casteless species, and determined their role in nestmate
96 recognition and defence. Specifically, we asked the following questions: (1) does the
97 response of bees to non-nestmates depend upon their proximity to nest odours; (2) do
98 guards and foragers respond differently to non-nestmates; and (3) do guards and foragers
99 differ in antennal morphology?

100

101 **Methods**

102 Colonies of *T. carbonaria* (n=5) used in our experiments were sourced from distinct and
103 unrelated wild colonies and raised in artificial hive boxes in suburban Brisbane, Australia.
104 Hive boxes contain a nest of around 8kg, similar to that of established wild colonies (Heard
105 2016). Foragers were caught in the air, using a clean plastic bag. Guards form a distinctive
106 ring at the nest entrance and were captured in a glass vial. Vials were used once only, to

107 eliminate contamination through volatile or cuticular odours. Our sample includes workers of
108 a range of ages because brood of *T. carbonaria* is produced continuously (Heard 2016).

109

110 Trials

111 A forager or guard (focal bee) was chosen from the holding container and placed in the test
112 arena with a stimulus bee that was either a forager nestmate or forager non-nestmate. Only
113 the behaviour of the focal bee was monitored.

114

115 The effect of social context was examined by employing two sets of assays: the first was
116 conducted in an enclosed glass container washed with hexane between trials (**Fig. 1a**); and
117 the second was conducted in a tube that was attached to the nest entrance (**Fig. 1b**). The
118 container in the first trial was located roughly 6-8 metres from the nest. In the second trial,
119 gauze was placed between the colony entrance and the test arena, allowing passage of
120 volatile chemical odours, but not bees, into the test arena. In both assays, bees were
121 allowed to acclimatize for one minute before being introduced to each other.

122

123 Trials only began once the bees had encountered each other, thereby accounting for any
124 effects of different sized containers. Each trial was then filmed (Sony DSCH90) for three
125 minutes. The digital recordings were replayed, and we noted the frequency of the following
126 behaviours of the focal bee: antennating or mutual touching of the antenna (typically
127 associated with mutual identification). Chasing, (one bee pursues the other, usually
128 accompanied by antennating on the abdomen of the escaping bee), a behaviour common in
129 *Apis mellifera* (Breed et al. 2004) and other meliponines (Inoue et al. 1999); and
130 grasping/fighting with mandibles and legs preventing either bee from escaping. This fighting
131 behaviour is often observed in this species and can involve a swarm of thousands of
132 workers (Heard 1996; Gloag et al. 2008; Cunningham et al. 2014).

133

134 Each of the four treatments were replicated with ten trials, using a randomized collection
135 of bees from across the five colonies. Colony identity and treatment were not confounded,
136 and the behavioural data were collected blind to task and nestmate status.

137

138 Morphological assays

139 After each trial, the focal bees were euthanized in a freezer. The antennae were removed
140 from the head and dehydrated through ethanol solutions of 75, 80, 85, 90, 95, and 100%.

141 Antenna were critically dried (Balzers CPD 030), then sputter coated with gold and

142 photographed in a scanning electron microscope (Phillips XL30 FEG). Sensilla density was

143 calculated across the middle two segments (five and six) and the most distal segments (nine

144 and ten) of the antenna. We manually counted the number of sensilla present within three
145 quadrats drawn onto each segment using ImageJ v1.49 with the Cell Counter package
146 (Schneider et al. 2012). The value across the quadrats was averaged, giving a mean value
147 for density per 1000 μm^2 . Body size, measured by intertegular distance, and head width,
148 measured as the widest point of the head viewed from above, were also calculated in
149 ImageJ.

150

151 Statistical analysis

152 We calculated an aggression index, following (Gill et al. 2013):

$$\frac{\sum_i^3 (A_i \times f_i)}{n}$$

153 where A_i denotes the assigned aggression score for each behaviour i , f is the frequency of
154 the behaviour and n is the number of all behaviours recorded. The behaviours were
155 assigned the following scores: antennation – 1; chasing – 2; grasping/fighting – 3.

156 We included antennation behaviour in the index because it represents an important
157 component of nestmate discrimination (see Breed et al., 2004), and also because we
158 observed bees persistently chasing and antennating non-nestmates, suggesting that the
159 behaviour has functions beyond ‘exploratory’ behaviour. Aggression and density scores
160 were compared using mixed models, with colony as a random effect, in JMP (v12.1), and
161 pairwise comparisons for aggression were analysed using Tukey’s post hoc test.

162

163 Results

164 *Social context*

165 Bees in the sealed glass container displayed none of the behaviours observed in typical
166 worker-to-worker interactions in natural populations. Instead, the bees spent most of their
167 time attempting to locate a point of escape. In contrast, when the assay container was
168 connected to the nest, bees interacted frequently, and antennation, at a minimum, was
169 recorded during each trial, (see electronic supplementary material). There was a significant
170 difference in their behaviour across the four treatments ($F_{3, 41} = 65.5866$, $p < 0.001$). The
171 mean aggression scores of foragers and guards against test nestmate bees were low (**Fig.**
172 **2a**), and did not strongly differ between tasks (Tukey’s *post hoc* test, $p = 0.0557$). Conversely,
173 both focal guards and foragers reacted aggressively towards non-nestmate test bees, and
174 their scores were markedly different between tasks (Tukey’s *post hoc* test, $p < 0.001$), with
175 the mean aggression scores of guards 1.2 times greater than that of foragers.

176

177 The antennation frequency of focal foraging bees toward non-nestmate foragers (**Fig. 2b**)
178 was significantly higher than focal guard; the mean frequency of foragers is 1.9 times higher
179 than that of guards (Tukey's *post hoc* test, $p=0.0161$).

180

181 Morphological assays

182 The sensilla density of both foragers and guards increased distally along the length of the
183 antenna (**Fig. 3**). However, regardless of segment, sensilla density was significantly higher
184 in guards compared with foragers ($F_{(1,38)} = 5.0368$, $p=0.0369$). Guards are also significantly
185 larger than foragers. The intertegular distance of guards (**Fig. 4**) ($1.188\text{mm} \pm 0.0273$) is
186 significantly larger than that of foragers (1.109 ± 0.0197), a mean difference of approximately
187 7% ($t_{(18)} = 2.1001$, $p = 0.0302$). Comparative results of head width are also consistent with
188 intertegular distance; the heads of guards (**Fig. 5**) ($1.857\text{mm} \pm 0.0146$) are significantly
189 wider than that of foragers (1.787 ± 0.0147), a difference of approximately 4% ($t_{(18)} = 2.1009$,
190 $p = 0.0032$).

191

192 Discussion

193 The response of workers of *T. carbonaria* to conspecifics depends upon both social context
194 and task. When isolated from their nest, workers did not interact with each other, but they
195 displayed inquisitive and aggressive behaviour towards one another in the presence of nest
196 odours. In these encounters, guards were more aggressive than foragers, reflected by a
197 greater willingness to physically engage with intruders. Workers are not only behaviourally
198 distinct but morphologically different; guards are both larger, and have significantly greater
199 numbers of antennal sensilla than foragers.

200

201 Our experiments highlight the importance of nest context in eliciting defensive behaviour in
202 *T. carbonaria*. Workers are likely to be able to detect their own nests through chemical
203 odours from the nest material, which in meliponines, is often similar to the workers cuticular
204 profile (Leonhardt et al. 2011a). This may allow guards to readily compare the cuticular
205 profiles of returning workers against nest odour. In addition, workers in close proximity to the
206 nest may also respond to vibrational signals that may reveal nest identity (Hunt & Richard
207 2013). The importance of workers confining their defensive behaviour to the critical area of
208 the nest entrance may reflect the potential cost of recognition errors: experimentally induced
209 defensive swarm responses from a *T. carbonaria* colony revealed that one in five workers
210 engaged in fatal fighting had inadvertently coupled with a nestmate (Gloag et al. 2008). In
211 addition, these fighting swarms may last for several days, seriously affecting the ability of the
212 colony to forage. Given the cost of these antagonistic encounters, there is a strong

213 functional benefit to ensuring that defensive behaviour is context-specific. Our results may
214 help explain the surprisingly low levels of aggression towards non-nestmates in other bees,
215 revealed from trials in isolated arenas (Leonhardt et al. 2011b; Dew et al. 2014) but see
216 (Breed & Page 1991). Social context is also important in honey bees, whose defensive
217 behaviour is usually only expressed at the nest entrance and environmental or social cues
218 may be required to discriminate nestmates (Downs & Ratnieks 1999; Buchwald & Breed
219 2005). Similarly, gynes of *Polistes fasciatus* that are not exposed to their natal nest struggle
220 to discriminate between related and non-related females (Shellman & Gamboa 1982).
221 Future nestmate recognition studies should consider the role of social odours when
222 measuring aggression, or they may fail to capture the full range of behaviours performed in
223 nest defence (Breed 2003).

224
225 The consistent pattern of differential investment in antennal sensilla between guards and
226 foragers likely reflects differences in required capacity to detect signals. Guards are the
227 frontline defence of the nests of meliponine bees, and threats of usurpation from
228 neighbouring conspecific colonies are common (Cunningham et al. 2014; Grüter et al. 2016).
229 In bumblebees, larger workers have a higher density of olfactory antennal sensilla and
230 greater odour sensitivity (Spaethe et al. 2007). Similarly, higher sensilla density may allow *T.*
231 *carbonaria* guards to identify intruders more quickly and or accurately than foragers,
232 especially if these sensilla contain receptors for nest recognition signals. This interpretation
233 of a link between sensilla density and efficient signal detection is consistent with reported
234 differences in the density of sensilla between social and solitary bees, which presumably
235 reflect a greater investment in antennal receptor organs in social species (Wittwer et al.
236 2017).

237
238 Constraining the definition of worker castes to obvious gross polymorphisms may be
239 unhelpful, as differences in finer morphological structures may create 'cryptic castes' within
240 worker populations. We use 'cryptic' to indicate that the morphological differences are simply
241 less obvious than in 'conventional' castes – in a manner analogous to 'cryptic' female choice
242 and 'cryptic' species. Although foragers in our assays chased and antennated more often
243 than guards, only guards attacked non-nestmate intruders. Soldier or guard castes of
244 polymorphic social insects are also typically larger and have elevated levels of aggression
245 (Jandt & Dornhaus 2009; Kamhi et al. 2015). A larger body and greater head width may
246 benefit guards, who are most likely to engage intruders in physical contests, where a large
247 body size is likely to be useful. Our results are consistent with a recent study documenting
248 worker differentiation in ten other stingless bee species (Grüter et al. 2017), where size and
249 colour dimorphism is associated with previously unknown guard castes. Age polyethism

250 (Wilson 1971) is not precluded as a driver of task allocation in *T. carbonaria*: younger
251 workers may remain in the nest, engaging in in-nest duties, subsequently adopting either
252 foraging or guarding tasks depending upon their sensilla density. Indeed, some guards may
253 subsequently become foragers, perhaps if their antennae are damaged (see Gill et al.,
254 2012). Accordingly, we predict far greater variation in the body and antennal size of younger
255 workers confined to the nest.

256

257 Variation in antennal morphology in these previously unnoticed cryptic castes allows *T.*
258 *carbonaria* to produce workers optimally positioned to defend their colonies. Odour mediated
259 aggression ensures workers maintain behavioural plasticity, thereby giving the colony a
260 flexible and efficient response to threats.

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375 **Figure 1 – Test arenas used in nestmate recognition trials (a)** Bees were assayed in a
376 partitioned glass container, which was cleaned of odours between trials and located
377 between 6 and 8 metres from the nest. Each bee was given one minute to acclimatize
378 before the petition was raised and the trial began. **(b)** Second-round assays were performed
379 in a tube attached to the nest entrance, separated from the entrance by gauze, ensuring the
380 test bees did not encounter an erroneous worker. Again the test bee was given one minute
381 to acclimatize before the trial began.

382

383 **Figure 2 – Variation in aggression and antennation frequency of focal bees towards**
384 **nestmates and intruders in nest entrance trials. (a)** Against nestmates aggression was
385 low and similar between guards and foragers. Against intruders, guards were significantly
386 more aggressive than foragers (Tukey's *post hoc* test, $p < 0.001$). **(b)** Foragers antennated
387 on the antenna and abdomen of intruders significantly more often than the other treatments,
388 indicative of recognition but displaying an unwillingness to fight (Tukey's *post hoc* test,
389 $p = 0.0161$). N = 10 for each trial. (Fn – forager nestmate, Fnn – forager non-nestmate, Gn –
390 guard nestmate, Gnn – Guard non-nestmate)

391

392 **Figure 3 – Mean (\pm standard error) sensilla density on antennomeres five, six, nine**
393 **and ten of *T. carbonaria* foragers and guards.** Consistent with most reported
394 Hymenopteran, density increases distally along the antenna in all workers. However, density
395 in guards is significantly higher than foragers, particularly on the most distal region of the
396 antenna ($F_{(1,1)} = 5.0368$, $p = 0.0369$, $n = 10$). Bars are marked with standard error.

397

398 **Figure 4 - Guards are consistently larger than foragers, as measured by intertegular**
399 **distance.** Mean size of guards is $1.188\text{mm} \pm 0.0273$, which is significantly greater than that
400 of foragers, with a mean of $1.109\text{mm} \pm 0.0197$. White circles represent individual data points.
401 N = 10.

402

403 **Figure 5 – Head width of guards is also consistently larger than that of foragers.** Mean
404 head width of guards is $1.857\text{mm} \pm 0.0146$, which is significantly greater than that of foragers
405 with a mean of 1.787 ± 0.0147 . White circles represent individual data points. N = 10.



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