RESEARCH ARTICLE

Context affects nestmate recognition errors in honey bees and stingless bees

Margaret J. Couvillon[1,*], Francisca H. I. D. Segers[1,2,3], Roseanne Cooper-Bowman[1], Gemma Truslove[1],
Daniela L. Nascimento[2], Fabio S. Nascimento[2] and Francis L. W. Ratnieks[1]

[1]Laboratory of Apiculture and Social Insects, School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, UK,
[2]Departamento de Biologia, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo,
CEP 14040-901, Ribeirão Preto, SP, Brazil and [3]Laboratoire d'Ethologie Expérimentale et Comparée, EA4443, Université Paris 13,
Sorbonne Paris Cité, 99 Avenue J. B. Clément, 93430 Villetaneuse, France

*Author for correspondence (M.Couvillon@Sussex.ac.uk)

SUMMARY

Nestmate recognition studies, where a discriminator first recognises and then behaviourally discriminates (accepts/rejects) another individual, have used a variety of methodologies and contexts. This is potentially problematic because recognition errors in discrimination behaviour are predicted to be context-dependent. Here we compare the recognition decisions (accept/reject) of discriminators in two eusocial bees, *Apis mellifera* and *Tetragonisca angustula*, under different contexts. These contexts include natural guards at the hive entrance (control); natural guards held in plastic test arenas away from the hive entrance that vary either in the presence or absence of colony odour or the presence or absence of an additional nestmate discriminator; and, for the honey bee, the inside of the nest. For both honey bee and stingless bee guards, total recognition errors of behavioural discrimination made by guards (% nestmates rejected + % non-nestmates accepted) are much lower at the colony entrance (honey bee: 30.9%; stingless bee: 33.3%) than in the test arenas (honey bee: 60–86%; stingless bee: 61–81%; *P*<0.001 for both). Within the test arenas, the presence of colony odour specifically reduced the total recognition errors in honey bees, although this reduction still fell short of bringing error levels down to what was found at the colony entrance. Lastly, in honey bees, the data show that the in-nest collective behavioural discrimination by *ca.* 30 workers that contact an intruder is insufficient to achieve error-free recognition and is not as effective as the discrimination by guards at the entrance. Overall, these data demonstrate that context is a significant factor in a discriminators' ability to make appropriate recognition decisions, and should be considered when designing recognition study methodologies.

Key words: *Apis mellifera*, *Tetragonisca angustula*, acceptance threshold, eusocial bee, social insects.

INTRODUCTION

Although nestmate recognition in social insects has been widely studied, previous work has used a variety of methodologies and social contexts. Bioassays to quantify the differential treatment of nestmates *versus* non-nestmates range from presenting natural guards at colony entrances with incoming foragers to observing isolated non-guard individuals in containers and presenting them with individuals who have been isolated from a colony since eclosion. The use of these highly artificial contexts has the advantage of affording greater control of the experimental conditions, and such studies have generated significant advances in our understanding of group-level behavioural discrimination (Breed, 1983; Getz and Smith, 1983; Breed et al., 1988; Buchwald and Breed, 2005). However, this may be at the expense of relevance to the actual challenges faced by discriminators in nestmate recognition.

The theory of optimal acceptance thresholds predicts that a discriminator will behave more or less permissively depending upon the context, by trading off one error (reject nestmate) for the other (accept non-nestmate) to minimize the total cost of all errors (Reeve, 1989; Couvillon et al., 2009; Jones et al., 2012). One such context that affects recognition behaviour is the perceived threat: for example, when honey bees face increased levels of conspecific robbing, the guards' threshold becomes less permissive, resulting in a lower

proportion of both non-nestmates and nestmates being accepted (Downs and Ratnieks, 2000; Couvillon et al., 2008). Another context is perceived proximity of the colony; paper wasps' aggression varies depending on the presence of a nestmate or a familiar nest fragment (Starks et al., 1998). However, in spite of these data, many previous studies that quantify recognition behaviour do not account for context. Additionally, results obtained in different contexts are often compared across studies (Johnson et al., 2011). If context is not considered, such comparisons are rendered meaningless.

In this study we carried out bioassays on nestmate recognition by guards in two species of eusocial bees, the honey bee *Apis mellifera* Linnaeus 1758 and the stingless bee *Tetragonisca angustula* (Latreille 1825). We systematically varied the naturalness of the context from fully natural (guards at nest entrances) through different degrees of unnatural (single *versus* two guards in a container, with and without colony odour). If there is no effect of context on guarding recognition behaviour (accept/reject an introduced bee), we would expect guards to make similar recognition errors under all treatments. Alternatively, if context does play a role, then we would expect guards to make different recognition errors between contexts. Specifically, we predicted that the lowest total recognition errors would be at the natural nest entrance, with the other contexts varying in their degree of error.

MATERIALS AND METHODS
Study organisms

We studied the effect of context in two species of bees, the honey bee *Apis mellifera* and the stingless bee *Tetragonisca angustula*. Both are highly eusocial; recent evidence suggests that they belong to the same eusocial clade but represent two independent origins of large colonies within this clade (Kawakita et al., 2008; Whitfield et al., 2008; Rasmussen and Cameron, 2010; Cardinal and Danforth, 2011). Therefore, it is likely that the development of highly social behaviours such as nest defence is also of independent origin. Recognition behaviour in both bees has been widely studied, and both have natural nest entrances with guards present. Honey bee guards represent a temporal subcaste of workers who perform their recognition duties for 1–1.5 days before transitioning into another duty (Breed and Rogers, 1991). *Tetragonisca angustula* also possesses highly developed recognition behaviour: guards stand on the outside of the wax entrance tube ('standing guards') to examine incomers to the hive, similar to *A. mellifera*. Additionally, *T. angustula* also have 'hovering guards', which fly in front of the entrance and detect allospecific intruders from a wider distance (van Zweden et al., 2011). For this experiment we only used standing guards, as their primary function is to distinguish and behaviourally discriminate between conspecific non-nestmates and nestmates. Guards in *T. angustula* (both standing and hovering) are a morphologically distinct caste (Grüter et al., 2012) and guard for longer than in honey bees.

We studied honey bees at the Laboratory of Apiculture and Social Insects, on the campus of the University of Sussex in Brighton, UK. Data were collected from September to November 2011 on non-rainy days between 10:00 and 16:00 h when the outside temperature was >10°C and there was foraging activity. Colonies studied were of mixed European race, predominantly the native subspecies *Apis mellifera mellifera*, and were queenright with ~5000 workers plus brood. Each colony was housed in glass-walled observation hives with three deep frames or one deep plus three medium frames. Each had a tube leading through the laboratory wall to allow foraging. We fitted a wooden entrance platform (10×10 cm) to the outside end of each tube. Guards naturally patrolled the platform, allowing their behaviour and interactions with experimentally introduced conspecifics to be observed. We used four discriminator colonies (A–D) in the experiment. Colony E was the source of non-nestmates.

We studied stingless bees in a meliponiary adjacent to the Laboratory of Behaviour and Ecology of Social Insects at the University of São Paulo at Riberão Preto, São Paulo State, Brazil. We gathered data in March 2012 on non-rainy days between 10:00 and 18:00 h. Our study colonies were healthy, queenright and had ~3000–10,000 workers plus brood. Colonies were housed in either wooden or plastic hives. Each colony had a wax entrance tube built by the bees. Guards stood around the tip of the entrance tube [see fig. 1A in Grüter et al. (Grüter et al., 2012)], where their behaviour and interactions with experimentally introduced conspecifics could be observed. We used six discriminator colonies (F–K) plus three additional colonies (L–N) as sources of non-nestmates. Halfway through the experiment, colony M was replaced by colony N because M had insufficient bees. Non-nestmates from colonies L and M/N were used randomly in equal proportions, and donor colony was included as a random effect in all models (see Data analysis) to control for any differences.

Determining recognition errors at the natural hive entrance

We used a behavioural assay of discrimination by natural entrance guards, as previously used in both honey bees (Downs and Ratnieks,

2000; Couvillon et al., 2008; Couvillon et al., 2009) and *T. angustula* (Kärcher and Ratnieks, 2009; van Zweden et al., 2011; Jones et al., 2012) and other stingless bees (Couvillon and Ratnieks, 2008). For honey bees, we captured returning foragers without pollen from colonies A–E in plastic vials specific for each colony; for *T. angustula*, we captured departing foragers by letting them fly into a plastic vial designated for that colony (F–N). For both species, we chilled bees until they could move but not fly. Guards were identified by their characteristic posture of standing on the entrance tube or platform with forward-facing antennae (Butler and Free, 1951; Couvillon et al., 2008) and other behavioural idiosyncrasies, specifically not flying away and antennating or being aggressive towards bees entering the hive.

We transferred one bee, either a nestmate or non-nestmate, at a time to the entrance of a discriminator hive using either forceps (honey bee) or a wooden toothpick (stingless bee). We observed the behaviour of the first guard to contact the introduced bee. The interaction was scored as rejection if the guard stung, grappled, pulled or bit the introduced bee. The interaction was scored as acceptance if the introduced bee was left alone after being inspected by a guard or if the introduced bee remained uninspected after 3 min and was allowed to enter the hive without inspection. In most cases (>95%), introduced bees were immediately contacted by guards and were accepted or rejected. As recommended, the observer who scored the accept or reject was blind to the source of that bee, i.e. nestmate or non-nestmate (Gamboa et al., 1991).

Determining recognition errors in different unnatural contexts (Treatments 1–4)

In Treatment 1 (no colony odour/lone guard), a single guard from each discriminator hive was marked with a small dot of acrylic paint and transferred from the nest entrance to a testing arena, either a 9 cm diameter×1 cm high Petri dish (honey bee) or a 1.5 ml Eppendorf tube (*T. angustula*). As *T. angustula* is a much smaller bee, a smaller arena was used. A second bee, either a nestmate or non-nestmate, was collected as described above and introduced into the arena. The observer then scored their interaction as accept or reject by the guard using the same criteria as described above. In Treatment 2 (no odour/two guards), an additional marked guard was added to the sterile arena before testing; however, behaviour was scored based on the original marked guard's interaction with the introduced nestmate/non-nestmate.

Treatments 3 and 4 were similar to Treatments 1 and 2 except that the test arenas were also allowed to acquire the odour of the guard's colony. For the honey bees, we achieved this by placing a plastic box over an opening in each discriminator observation hive. Petri dishes were then placed into this box for at least 24 h prior to a trial, so that they were walked over by the colony's bees. At the end of each experimental day, the Petri dishes were returned to their box. For stingless bees, we placed Eppendorf tubes inside each discriminator hive on the wax involucrum that covers the brood chamber. Bees crawled over the tubes for at least 24 h before the start of the experiment and at the end of each experimental day the tubes were returned to their respective hives.

For honey bees, randomisation of treatment order was not possible because each hive only had two to three guards, on average, visible each day. Instead, the same guards were sequentially tested at the entrance and then in the four treatment contexts (1–4) with both a nestmate and a non-nestmate. This may potentially lead to increased rejection rates by a guard for both nestmates and non-nestmates, as discriminators are known to reject significantly more individuals when there is an increased frequency of encountering a

non-nestmate (Reeve, 1989; Downs and Ratnieks, 2000; Couvillon et al., 2008). However, this does not seem to be the case, as guards did not reject significantly more nestmates or non-nestmates from Treatments 1–4 (see below). In *T. angustula*, we were able to randomise the order of treatments because many more guards are available at any given time.

In all our analyses, we performed the necessary corrections for multiple observations on the same guard bee within a day (see below). Because of rapid turnover in guards each day (Breed and Rogers, 1991) and because guards used previously were marked, we therefore used new guards each experimental day. Guarding behaviour in all contexts (natural entrance plus Treatments 1–4) was tested on all days. In the arena treatments, we introduced 440 honey bees (55 nestmates and 55 non-nestmates per treatment, equally distributed over the four discriminator colonies) and 288 stingless bees (36 nestmates and 36 non-nestmates per treatment, equally distributed over the six discriminator colonies).

Determining in-hive recognition errors in *A. mellifera*
First contact (Treatment 5A)

Because the honey bees were housed in observation hives, we were able to observe the treatment of nestmates and non-nestmates introduced directly into the nest in addition to at the entrance. This was accomplished by replacing the glass in hives C and D with clear plastic, into which we drilled six circular holes, 3 cm in diameter. Into each hole we placed a 50 ml screw-top centrifuge tube, from which we cut off the conical bottom. The lid faced outwards, and we also cut a small slit into each tube, which allowed us to insert a piece of a plastic cup. For each experimental trial, a test bee was collected (as above), marked with a dot of paint and placed in the tube. The introduced bee was allowed to warm up in the holding area, at which stage we lifted the plastic piece so that the bee could enter the hive and interact with other bees. For comparison with Treatments 1–4, we scored the behaviour (accept/reject) of the first bee to contact the introduced bee using the criteria described above. As always, the scorer was blind to the source of the introduced bee. We were only able to perform Treatment 5A (and 5B; see below) on two of the four discriminator hives; however, as the results were so robust while controlling for colony effects by using generalized linear mixed effect models (GLMMs; see below), we include Treatment 5A in Fig. 1. We introduced 98 nestmate and 98 non-nestmate bees in Treatment 5A.

Because the *T. angustula* colonies were kept in box hives, we were not able to perform in-hive tests of nestmate recognition.

Cumulative contact over 3 min (Treatment 5B)

For our in-hive treatment, we also scored accept/reject based on how the introduced bee was being treated at minute 3. However, in almost all cases (>95%), if a rejection occurred within the 3 min, we would still be witnessing aggression by minute 3. We estimated that over the 3 min, each introduced bee was contacted by 20–40 in-hive bees. For this treatment, we followed 146 (75 nestmates and 71 non-nestmates) of the 196 bees from Treatment 5A.

Data analysis

All data analysis was performed in R 2.9.2 using the lmer function from the lme4 package (http://cran.r-project.org/web/packages/lme4/index.html). We used GLMMs with binomial error structure. Colony was always included as a random effect to control for non-independence of data. For the same reason, guard was nested within colony as a random effect when we made multiple observations on the same guard. For *T. angustula* we also added donor colony as a
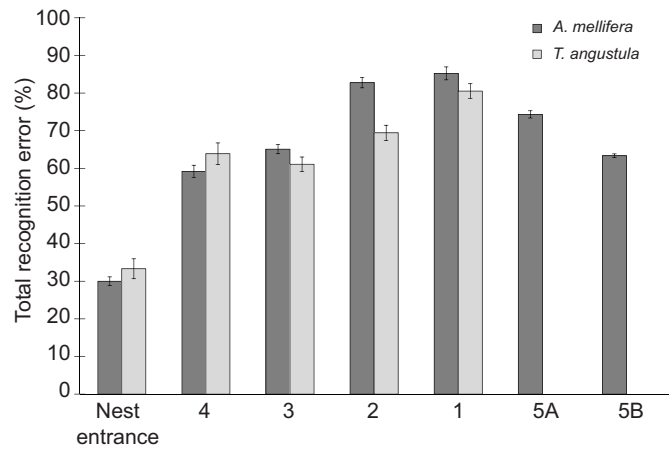

Fig. 1. Both the honey bee *Apis mellifera* and the stingless bee *Tetragonisca angustula* make the lowest amount of total recognition errors at the hive entrance compared with other contexts (Petri dish/Eppendorf with or without colony odour or an additional guard present). Total recognition error is acceptance error + rejection error, and error bars are s.e.m. between colonies. In-hive treatments 5A and 5B were only performed in honey bees.

random effect to the models to control for a potential difference in acceptance rate of non-nestmates from the three different donor colonies (L, M and N). We tested for the significance of two-way interactions by comparing models with and without the interaction using a likelihood ratio test (Zuur et al., 2009). Non-significant interactions were removed from the models, with the criterion set at 0.05. The main effects were always kept in the models.

We began by determining whether there was a difference in total recognition error (acceptance error + rejection error) made by guards in the natural context (hive entrance) *versus* the unnatural contexts (Treatments 1–4) [error (yes/no) ~ context, where the ~ indicates dependence; i.e. error, the response/dependent variable, is modelled against the factor of context, the independent variable] for both *A. mellifera* and *T. angustula*. For *A. mellifera*, we included an analysis comparing differences in total recognition errors made by guards in the natural context *versus* in-hive first contact (Treatment 5A) and in-hive cumulative contact (Treatment 5B).

We then determined whether there was a difference in guard acceptance rates of nestmates and non-nestmates in the natural *versus* unnatural contexts (acceptance ~ context×bee type) for both *A. mellifera* and *T. angustula*. The dependent variable of the GLMMs was guard response to the introduced bee (accept/reject).

Next, we explored the difference in acceptance rates of nestmates and non-nestmates between guards in the four unnatural contexts for both *A. mellifera* and *T. angustula*. In other words, how was the acceptance rate in the unnatural contexts influenced by guard number and the presence of colony odour? For this part of the analysis, we separated nestmates and non-nestmates to make the interpretation of the model terms more straightforward (acceptance ~ number of guards×odour presence). In every model the number of guards (one or two) was included as a categorical variable.

Lastly, we examined in *A. mellifera* how first contact of an introduced bee within the hive compared with cumulative contact after 3 min (acceptance ~ bee type×first contact *versus* cumulative contact) and how these in-hive treatments differed from the natural entrance (acceptance ~ bee type×natural entrance *versus* first/cumulative contact). When two measurements were performed on the same introduced bee (first contact and cumulative), the

introduced individuals were included as a random effect in the model.

As we performed multiple tests on the same data sets, we corrected for multiple testing. *P*-values that remained significant after the sequential Bonferroni correction are marked with an asterisk.

RESULTS

As expected, across all experimental contexts both honey bee and stingless bee guards were able behaviourally to distinguish nestmates from non-nestmates (stingless bees: bee type: $N=408$, $z=6.10$, $P<0.001*$; honey bees: $N=892$, $z=10.26$, $P<0.001*$; Fig. 2).

Total errors are lowest at the hive entrance in both species

There was a significant effect of context on the total recognition error probability (acceptance error + rejection error) made by guards in both *A. mellifera* ($N=550$, $z=4.12$, $P<0.001*$) and *T. angustula* ($N=408$, $z=3.47$, $P<0.001*$; Table 1, Fig. 1). Specifically, in both *A. mellifera* and *T. angustula*, total errors were lowest at the nest entrance (30.9 and 33.3%, respectively) and highest in the arena with a single guard and without colony odour (85.5 and 80.5%, respectively). Total errors in the other unnatural contexts (2 and 3) were between these two extremes, with Treatments 3 and 4 (colony odour present), at 60.0–65.5% for both bees, being closest to the natural nest entrance (see below for detailed analysis; Table 1, Fig. 1).

Guards make significantly more acceptance errors in non-natural contexts in both *A. mellifera* and *T. angustula*

We tested for overall differences in acceptance rate of introduced bees (nestmates and non-nestmates) in the natural (hive entrance) *versus* the unnatural contexts (Treatments 1–4) for *A. mellifera* and *T. angustula*.

For honey bees, there was a significant interaction between the type of bee that was introduced and the context ($N=550$, context×bee type: $\chi^2=10.56$, $P=0.001*$). To learn more about this interaction, we analysed the data separately for nestmates and non-nestmates. There was no significant difference between the acceptance rate of nestmates at natural entrances (90.9%) *versus* in Petri dishes (83.6–92.7%; $N=275$, context: $z=-0.10$, $P=0.92$; Fig. 2A, Table 1). In contrast, there was a significant difference for non-nestmates

(21.8% *versus* 43.6–78.2%; $N=275$, context: $z=5.22$, $P<0.001*$; Fig. 2A, Table 1).

Looking specifically at the unnatural contexts (Treatments 1–4), we next tested how the presence of an additional nestmate guard and colony odour influenced acceptance. Once again, we analysed data separately for nestmates and non-nestmates ($N=220$, for both models). Not surprisingly, given that there was no significant difference between the acceptance rate of nestmates at the natural entrance compared with the Petri dishes, there was no effect of either honey bee guard number or odour presence on the acceptance rate of nestmates (number of guards: $z=-1.15$, $P=0.25$; odour: $z=-1.15$, $P=0.25$; number of guards×odour: $\chi^2=0.92$, $P=0.34$). However, the presence of odour in the unnatural contexts decreased non-nestmate acceptance across Treatments 1–4 (odour: $z=-4.01$, $P<0.001*$), although acceptance remained higher than at the hive entrance. Guard number had no effect on the treatment of non-nestmates (number of guards: $z=-1.31$, $P=0.19$), nor was there a significant interaction between number of guards and odour presence (number of guards×odour: $\chi^2=0.65$, $P=0.42$).

In *T. angustula*, there was no significant interaction between the type of bee that was introduced and the context ($N=408$, context×bee type: $\chi^2=3.37$, $P=0.07$). There was a significant difference in the acceptance rates at the natural hive entrance (nestmate: 80.0%; non-nestmate: 13.3%) compared with the unnatural contexts (nestmates: 88.9–100%; non-nestmate: 58.3–69.4%; context: $z=6.59$, $P<0.001*$; bee type: $z=6.40$, $P<0.001*$; Fig. 2B, Table 1). This demonstrates that for *T. angustula*, guards became more globally accepting for both nestmates and non-nestmates in the treatments compared with the natural hive entrance. Thus in the unnatural contexts (Treatments 1–4), the guards made significantly more acceptance errors and fewer rejection errors, even though the reduction in rejection errors did not compensate in terms of significantly reducing total error (see above).

Looking only at the unnatural contexts (Treatments 1–4), we tested how the presence of an additional nestmate guard and colony odour influenced acceptance by *T. angustula*. Once again, we analysed data separately for nestmates and non-nestmates ($N=144$, for both models). There was no significant effect of guard number on nestmate acceptance in the Eppendorf tubes, nor was there a significant interaction between the number of guards and odours in
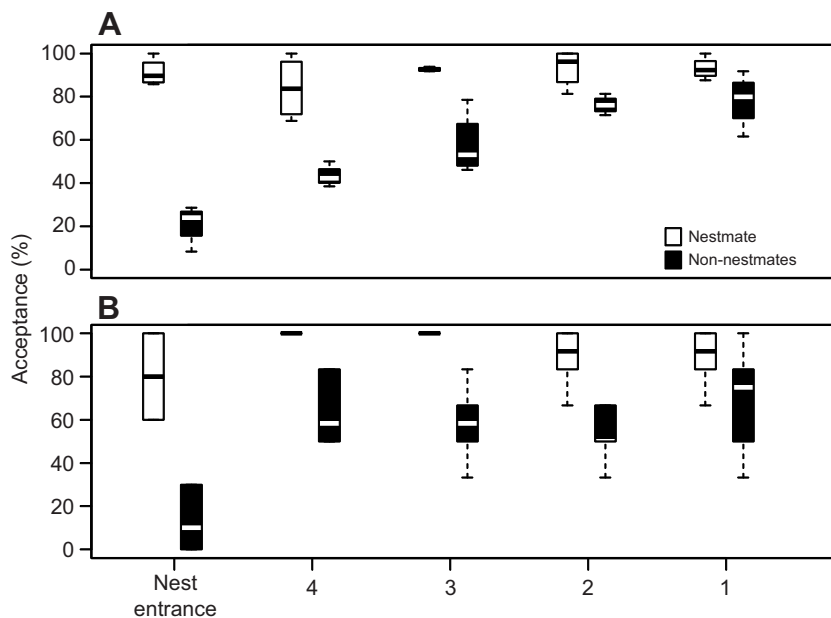

Fig. 2. Percentage acceptance of nestmates and non-nestmates at the natural entrance and in the four unnatural contexts for (A) *Apis mellifera* and (B) *Tetragonisca angustula*. The boxplots show medians, quartiles and 5th and 95th percentiles. Guards of both species became significantly more accepting of non-nestmates away from the natural entrance ($P<0.001$ for both) compared with the four unnatural contexts, and *T. angustula* became more accepting of nestmates as well ($P<0.001$; $P=0.92$ for *A. mellifera*). Within the unnatural contexts themselves, *A. mellifera* guards became significantly less accepting of non-nestmates if hive odour was present ($P<0.001$). Within the unnatural contexts, *T. angustula* guards tended to become more accepting of nestmates if hive odour was present ($P<0.04$, n.s. after Bonferroni correction). There was no significant difference in acceptance of non-nestmates in any of the Eppendorf treatments ($P>0.29$).

Table 1. Summary of the effect of context in the honey bee *Apis mellifera* and the stingless bee *Tetragonisca angustula*

		Guard bioassay				Colony bioassay	
Treatment	Control	1	2a	3	4	5A	5B
	Natural hive entrance	No odour/ 1 guard	No odour/ 2 guards	Odour/ 1 guard	Odour/ 2 guards	In-hive initial contact	In-hive cumulative contact
Apis mellifera							
Acceptance (%)							
Nestmate	90.9	92.7	92.7	92.7	83.6	95.9	80.3
Non-nestmate	21.8	78.2	76.4	58.2	43.6	73.5	46.5
Recognition error (%)							
Acceptance error	21.8	78.2	76.4	58.2	43.6	73.5	46.5
Rejection error	9.1	7.3	7.3	7.3	16.4	4.1	19.7
Total error	30.9	85.5	83.6	65.5	60.0	77.6	66.2
Tetragonisca angustula							
Acceptance (%)							
Nestmate	80.0	88.9	88.9	97.2	100.0		
Non-nestmate	13.3	69.4	58.3	58.3	63.9		
Recognition error (%)							
Acceptance error	13.3	69.4	58.3	58.3	63.9		
Rejection error	20.0	11.1	11.1	2.8	0.0		
Total error	33.3	80.5	69.4	61.1	63.9		

Acceptance is percent across all discriminator colonies by guards (or in-hive workers for 5A/5B) for nestmates and non-nestmates. There are two types of recognition error: acceptance error is the incorrect acceptance of non-nestmates and is equal to % acceptance of non-nestmates, and rejection error is the incorrect rejection of nestmates and is equal to 100 – % acceptance nestmates. Total error = acceptance error + rejection error per context.

the treatment of nestmates in the unnatural contexts (number of guards: z=0.35, P=0.73; number of guards×odour: χ^2=1.28, P=0.26). There was an increase of acceptance of nestmates in the presence of colony odours (z=2.03, P=0.04; Table 1, Fig. 2B); however, this effect was not significant after sequential Bonferroni correction. Additionally, there was no significant effect of hive odour and/or extra guard on the acceptance rate of non-nestmates, which remained erroneously high, through Treatments 1–4 (number of guards×odour: χ^2=1.14, P=0.29; number of guards: z=–0.24, P=0.81; odour: z=–0.30, P=0.77; Fig. 2B, Table 1).

Total recognition errors inside the *A. mellifera* nest remain high

Inside the nest, *A. mellifera* workers were able behaviourally to distinguish nestmates from non-nestmates, both at first contact and after cumulative contact (N=342, bee type: z=2.78, P=0.01*; Fig. 1, Table 1). Both nestmates and non-nestmates were significantly more likely to be rejected after being in the hive for 3 min compared with the acceptance rate at first contact (first contact *versus* cumulative contact: z=–5.92, P<0.001*; Fig. 1). There was no significant interaction between type of introduced bee and in-hive treatment [bee type×(first contact *versus* cumulative): χ^2=0.03, P=0.85].

When comparing acceptance at the natural entrance with first contact within the hive, there was a nearly significant interaction between type of introduced bee and context (natural entrance *versus* first contact within hive) (N=306, bee type×context: χ^2=3.07, P=0.08). Therefore, we divided the data and tested nestmates and non-nestmates separately. Nestmates had the same chance of being accepted at the entrance as at their first contact within the hive (N=153, context: z=–1.23, P=0.22; Table 1); in contrast, non-nestmates were significantly less likely to be accepted at the nest entrance by a guard than by the first in-hive worker bee (N=153, context: z=–5.76, P<0.001*; Fig. 1, Table 1). Therefore, the first natural entrance guard to contact a non-nestmate was significantly better at correctly rejecting compared with the first in-hive worker.

When comparing the acceptance rate at the natural entrance with the acceptance rate at minute 3 of cumulative contact, there was an interaction between type of introduced bee and experimental context (N=256, bee type×context: χ^2=8.71, P=0.003*). Thus, again the model was performed separately for nestmates and non-nestmates. Nestmates had the same chance of being accepted at the natural entrance as they were at minute 3 of in-hive cumulative contact (N=126, context: z=–1.57, P=0.12; Fig. 1, Table 1), although there was a trend for them to be more rejected. Lastly, non-nestmates were significantly more likely to be incorrectly accepted by the cumulative discrimination of in-hive bees over 3 min compared with the first contact of a guard at the nest entrance (N=130, context: z=2.58, P=0.01*; Fig. 1, Table 1). Therefore, even at 3 min and with the collective discrimination of *ca.* 30 worker bees, in-hive acceptance errors of non-nestmates remain significantly higher compared with acceptance errors at the natural hive entrance.

Total recognition errors inside the hive with first contact and cumulative contact were both significantly higher (natural entrance *versus* first contact, N=306, z=–4.12, P<0.001*; natural entrance *versus* cumulative contact, N=256, z=3.00, P=0.003*) than total recognition errors at the entrance.

DISCUSSION

Our results clearly show that context has a significant effect on nestmate recognition in both the honey bee *Apis mellifera* and the stingless bee *Tetragonisca angustula*. Total errors in behavioural discrimination made by guards were much lower in the natural context – the colony entrance – than in test arenas and were highest in the most unnatural context – a single guard in an arena lacking colony odour. Removing guards from the hive entrance seems to result in a much higher acceptance of non-nestmates (for both *A. mellifera* and *T. angustula*). Acceptance of nestmates was less affected, and was only significant in *T. angustula*, most likely because nestmate rejection by guards was not high at the nest entrance in the first place. Our results also show that introducing honey bees inside the hive away from the entrance and its dedicated guards also represents an unnatural recognition context, where recognition errors, even over 3 min and with the cumulative contact of 20–40 worker bees, are significantly higher than at the hive entrance. Across all treatments,

the 'best' unnatural context still resulted in more than double the total recognition errors made by guards stationed at the nest entrance.

In honey bees, adding back odour cues in unnatural contexts in Treatments 1–4 significantly improved a guard's ability to discriminate and to reject a non-nestmate ($P<0.001$), although the presence of an additional nestmate guard (social cue) did not help ($P=0.19$). Neither social ($P=0.25$) nor odour ($P=0.25$) cues affected guard recognition of a nestmate in Treatments 1–4, which did not significantly differ from the nest entrance ($P=0.92$). Of course, we have no way of knowing whether the odour in the arena is as strong as what a guard would experience at the nest entrance. However, these data still suggest that when it comes to rejecting non-nestmates correctly, honey bee guards are more attuned to odour indicating that the nest is nearby than they are to the presence of another nestmate guard. Recognition in social insects is generally assumed to be olfactory in nature (Getz, 1982; Lacy and Sherman, 1983; Breed et al., 2004a; Couvillon et al., 2009; Nascimento and Nascimento, 2012), so it stands to reason that odours would play an important role in signalling context.

In contrast, neither odour ($P=0.77$) nor an additional nestmate guard ($P=0.81$) significantly improved the ability of *T. angustula* guards to recognise and to reject non-nestmates in the unnatural contexts in Treatments 1–4. The presence of guards had a non-significant effect on the acceptance of nestmates ($P=0.73$) in the unnatural contexts. There was a trend towards an increase in acceptance rates of nestmates in Eppendorf tubes ($P=0.04$ before Bonferroni) in the presence of odours, which was no longer significant after correcting for multiple testing. This trend is probably best understood as the shift in the stingless bee guards' acceptance threshold in unnatural contexts to being more globally permissive. So in this circumstance, given the additional increased acceptance of non-nestmates through these treatments, this particular significant result is best seen as yet another example of careless guarding. Total recognition errors remain very high (more than twice that at the natural nest entrance). More importantly, the overall message is maintained. In both *T. angustula* and honey bees, the hive entrance is the best context for minimum total recognition errors.

Inside the honey bee hive, away from the nest entrance and specialist guards, both the first worker to contact the introduced bee and the cumulative contact over 3 min result in discrimination between nestmates and non-nestmates. Looking closer, nestmate acceptance did not significantly change between the nest entrance (91%) and in-hive first contact (96%, $P=0.22$) or in-hive cumulative contact (80%, $P=0.12$). However, non-nestmate acceptance was significantly different between nest entrance (22%) and in-hive first contact (74%, $P<0.001$) and in-hive cumulative contact (47%, $P=0.01$), which is similar to results from Treatment 4 (a Petri dish containing colony odours and two guards, 43.6%). In fact, the in-hive worker total recognition errors, at 78% for initial and 66% for cumulative contact, were more than twice the total recognition error at the hive entrance (31%, $P<0.001$). Therefore, even though rejection of non-nestmates was greater over 3 min compared with the initial contact, it remains ineffectual. Previous modelling work has predicted that, inside the hive, near-perfect recognition could be achieved through the collective process of multiple contacts between an introduced bee and that colony's worker bees (Johnson et al., 2011; Johnson et al., 2012). The model suggested that even if per-encounter (initial) rejection rate of a non-nestmate is 20%, then after just 21 encounters, the overall probability of acceptance of the non-nestmate should be <1% (Johnson et al., 2011). However, this is

not what we found. Workers that are not dedicated guards are not as adept at recognition as guards at the nest entrance, which is not surprising as guards represent a behaviourally and physiologically distinct subcaste (Breed et al., 1990). The lower acceptance, including of nestmates, can be understood as non-independent errors (Couvillon et al., 2012). Sometimes a nestmate's cues closely resemble those of a non-nestmate, and if a worker responds to this resemblance and directs aggression, then it is likely that the next in-hive worker will do the same.

Context is known to be important in recognition in many social insects (Hölldobler and Lumsden, 1980; Starks et al., 1998; D'Ettorre et al., 2004; Buczkowski and Silverman, 2005; Bos et al., 2010; van Zweden and d'Ettorre, 2010). For example, nest proximity seems to serve as an important context cue, and previous studies have demonstrated that discriminator aggression increases in a step-wise fashion with increasing nest proximity (Gamboa et al., 1991; Venkataraman and Gadagkar, 1992; Starks et al., 1998). The importance of context may even help explain unusual data, especially negative results. In a study in *Tetragonilla collina*, a discriminator bee was transferred from her colony into a clean Petri dish and presented with a dead bee from her colony, another colony within the same aggregation, or from a foreign aggregation (Leonhardt et al., 2011). The discriminator displayed almost a complete lack of aggression and earned the title 'unusually peaceful bee'. It may be that the non-response of the guard was due to her being removed from the colony entrance, her being tested with dead introduced bees, or an interaction of both unnatural contexts. In general, the issue is further complicated because some methodologies (e.g. staging interactions in vials or cardboard containers) do seem to work in that discriminators do direct aggression more towards non-nestmates to varying degrees (Breed, 1983; Breed et al., 1988; Breed et al., 2004a; Breed et al., 2004b; Buchwald and Breed, 2005). Indeed, some previous work invoked different methodologies (Breed et al., 2007) to explain a lack of aggression where one may be expected. What our work demonstrates is that even when discriminators are still able to differentiate nestmates from non-nestmates, as in the arenas, this unnatural context does affect the level and type of recognition errors made and may make the interpretation of results difficult.

That context should be accounted for in behavioural studies is not surprising. In a recent tribute to Paul MacLean, authors note that part of his legacy from his work on rat neuroethology was his attention to the social and environmental stimuli of an environment that must be incorporated into any meaningful laboratory work (Blanchard and Blanchard, 2003). For mammalian work, it has long been accepted that moving an animal from the context in which it normally behaves into a novel context will reduce performance (Burman and Mendl, 1999). Now we see the same is true for nestmate recognition of social bees.

Although our data demonstrate that both honey bee and stingless bee guards make more acceptance errors away from the nest entrance, it is unclear what specific mechanisms are responsible for the increase. It may be that errors are high in unnatural contexts because guards are missing the salient cues necessary to discriminate effectively. Alternatively, it may be that guards away from the nest decrease aggressive behaviour (even if they still are able to discriminate) because the cost of an aggressive act, which may be reciprocated, is not worthwhile if there is nothing nearby to guard (i.e. a nest, its resources, brood and nestmates). Lastly, it may be that removing guards from their setting confuses them such that they no longer behave appropriately.

AUTHOR CONTRIBUTIONS

M.J.C., F.H.I.D.S., F.L.W.R. and F.S.N. helped design the experiment. M.J.C., R.C.-B. and G.T. collected honey bee data. F.H.I.D.S. and D.L.N. collected stingless bee data. F.H.I.D.S. analysed the data, and M.J.C. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

FUNDING

REFERENCES

Blanchard, R. J. and Blanchard, D. C. (2003). Bringing natural behaviors into the laboratory: a tribute to Paul MacLean. *Physiol. Behav.* **79**, 515-524.

Bos, N., Guerrieri, F. J. and d'Ettorre, P. (2010). Significance of chemical recognition cues is context dependent in ants. *Anim. Behav.* **80**, 839-844.

Breed, M. D. (1983). Nestmate recognition in honey bees. *Anim. Behav.* **31**, 86-91.

Breed, M. D. and Rogers, K. B. (1991). The behavioral genetics of colony defense in honeybees: genetic variability for guarding behavior. *Behav. Genet.* **21**, 295-303.

Breed, M. D., Williams, K. R. and Fewell, J. H. (1988). Comb wax mediates the acquisition of nest-mate recognition cues in honey bees. *Proc. Natl. Acad. Sci. USA* **85**, 8766-8769.

Breed, M. D., Robinson, G. E. and Page, R. E. (1990). Division of labor during honey bee colony defense. *Behav. Ecol. Sociobiol.* **27**, 395-401.

Breed, M. D., Diaz, P. H. and Lucero, K. D. (2004a). Olfactory information processing in honeybee, *Apis mellifera*, nestmate recognition. *Anim. Behav.* **68**, 921-928.

Breed, M. D., Guzmán-Novoa, E. and Hunt, G. J. (2004b). Defensive behavior of honey bees: organization, genetics, and comparisons with other bees. *Annu. Rev. Entomol.* **49**, 271-298.

Breed, M. D., Deng, X.-B. and Buchwald, R. (2007). Comparative nestmate recognition in Asian honey bees, *Apis florea*, *Apis andreniformis*, *Apis dorsata* and *Apis cerana*. *Apidologie (Celle)* **38**, 411-418.

Buchwald, R. and Breed, M. D. (2005). Nestmate recognition cues in a stingless bee, *Trigona fulviventris*. *Anim. Behav.* **70**, 1331-1337.

Buczkowski, G. and Silverman, J. (2005). Context-dependent nestmate discrimination and the effect of action thresholds on exogenous cue recognition in the Argentine ant. *Anim. Behav.* **69**, 741-749.

Burman, O. H. P. and Mendl, M. (1999). The effects of environmental context on laboratory rat social recognition. *Anim. Behav.* **58**, 629-634.

Butler, C. B. and Free, J. B. (1951). The behaviour of worker honeybees at the hive entrance. *Behaviour* **4**, 262-292.

Cardinal, S. and Danforth, B. N. (2011). The antiquity and evolutionary history of social behavior in bees. *PLoS ONE* **6**, e21086.

Couvillon, M. J. and Ratnieks, F. L. W. (2008). Odour transfer in stingless bee marmelada (*Frieseomelitta varia*) demonstrates that entrance guards use an 'undesirable–absent' recogniton system. *Behav. Ecol. Sociobiol.* **62**, 1099-1105.

Couvillon, M. J., Robinson, E. J. H., Atkinson, B., Child, L., Dent, K. R. and Ratnieks, F. L. W. (2008). En garde: rapid shifts in honey bee (*Apis mellifera*) guarding behavior is triggered by onslaught of conspecific intruders. *Anim. Behav.* **76**, 1653-1658.

Couvillon, M. J., Roy, G. G. F. and Ratnieks, F. L. W. (2009). Recognition errors by honey bee (*Apis mellifera*) guards demonstrate overlapping cues in conspecific recognition. *J. Apic. Res.* **48**, 225-232.

Couvillon, M., van Zweden, J. and Ratnieks, F. (2012). Model of collective decision-making in nestmate recognition fails to account for individual discriminator responses and non-independent discriminator errors. *Behav. Ecol. Sociobiol.* **66**, 339-341.

D'Ettorre, P., Brunner, E., Wenseleers, T. and Heinze, J. (2004). Knowing your enemies: seasonal dynamics of host-social parasite recognition. *Naturwissenschaften* **91**, 594-597.

Downs, S. G. and Ratnieks, F. L. W. (2000). Adaptive shifts in honey bee (*Apis mellifera* L.) guarding behavior support predictions of the acceptance threshold model. *Behav. Ecol.* **11**, 326-333.

Gamboa, G., Reeve, H. and Holmes, W. (1991). Conceptual issues and methodology in kin-recognition research – a critical discussion. *Ethology* **88**, 109-127.

Getz, W. M. (1982). An analysis of learned kin recognition in Hymenoptera. *J. Theor. Biol.* **99**, 585-597.

Getz, W. M. and Smith, K. B. (1983). Genetic kin recognition: honey bees discriminate between full and half sisters. *Nature* **302**, 147-148.

Grüter, C., Menezes, C., Imperatriz-Fonseca, V. L. and Ratnieks, F. L. W. (2012). A morphologically specialized soldier caste improves colony defense in a neotropical eusocial bee. *Proc. Natl. Acad. Sci. USA* **109**, 1182-1186.

Hölldobler, B. and Lumsden, C. J. (1980). Territorial strategies in ants. *Science* **210**, 732-739.

Johnson, B. R., van Wilgenburg, E. and Tsutsui, N. D. (2011). Nestmate recognition in social insects: overcoming physiological constraints with collective decision making. *Behav. Ecol. Sociobiol.* **65**, 935-944.

Johnson, B. R., Wilgenburg, E. and Tsutsui, N. (2012). Nestmate recognition in social insects is sometimes more complex than an individual based decision to accept or reject. *Behav. Ecol. Sociobiol.* **66**, 343-346.

Jones, S., van Zweden, J., Grüter, C., Menezes, C., Alves, D., Nunes-Silva, P., Czaczkes, T., Imperatriz-Fonseca, V. and Ratnieks, F. (2012). The role of wax and resin in the nestmate recognition system of a stingless bee, *Tetragonisca angustula*. *Behav. Ecol. Sociobiol.* **66**, 1-12.

Kärcher, M. and Ratnieks, F. L. W. (2009). Standing and hovering guards of the stingless bee *Tetragonisca angustula* complement each other in entrance guarding and intruder recognition. *J. Apic. Res.* **48**, 209-214.

Kawakita, A., Ascher, J. S., Sota, T., Kato, M. and Roubik, D. W. (2008). Phylogenetic analysis of the corbiculate bee tribes based on 12 nuclear protein-coding genes (Hymenoptera: Apoidea: Apidae). *Apidologie (Celle)* **39**, 163-175.

Lacy, R. C. and Sherman, P. W. (1983). Kin recognition by phenotype matching. *Am. Nat.* **121**, 489-512.

Leonhardt, S. D., Form, S., Blüthgen, N., Schmitt, T. and Feldhaar, H. (2011). Genetic relatedness and chemical profiles in an unusually peaceful eusocial bee. *J. Chem. Ecol.* **37**, 1117-1126.

Nascimento, D. L. and Nascimento, F. S. (2012). Acceptance threshold hypothesis is supported by chemical similarity of cuticular hydrocarbons in a stingless bee, *Melipona asilvai*. *J. Chem. Ecol.* **38**, 1432-1440.

Rasmussen, C. and Cameron, S. A. (2010). Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. *Biol. J. Linn. Soc. Lond.* **99**, 206-232.

Reeve, H. K. (1989). The evolution of conspecific acceptance thresholds. *Am. Nat.* **133**, 407-435.

Starks, P. T., Fischer, D. J., Watson, R. E., Melikian, G. L. and Nath, S. D. (1998). Context-dependent nestmate discrimination in the paper wasp, *Polistes dominulus*: a critical test of the optimal acceptance threshold model. *Anim. Behav.* **56**, 449-458.

van Zweden, J. and d'Ettorre, P. (2010). Nestmate recognition in social insects and the role of hydrocarbons. In *Insect Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (ed. G. J. Blomquist and A. Bagnères), pp. 222-243. Cambridge: Cambridge University Press.

van Zweden, J., Grüter, C., Jones, S. and Ratnieks, F. (2011). Hovering guards of the stingless bee *Tetragonisca angustula* increase colony defensive perimeter as shown by intra- and inter-specific comparisons. *Behav. Ecol. Sociobiol.* **65**, 1277-1282.

Venkataraman, A. and Gadagkar, R. (1992). Kin recognition in a semi-natural context: behaviour towards foreign conspecifics in the social wasp *Ropalidia marginata* (Lep.) (Hymenoptera: Vespidae). *Insectes Soc.* **39**, 285-299.

Whitfield, J. B., Cameron, S. A., Huson, D. H. and Steel, M. A. (2008). Filtered Z-closure supernetworks for extracting and visualizing recurrent signal from incongruent gene trees. *Syst. Biol.* **57**, 939-947.

Zuur, A. F., Ieno, E. N., Walker, N., Saveliev, A. A. and Smith, G. M. (2009). *Mixed Effects Models and Extensions in Ecology with R*. New York, NY: Springer.