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ORIGINAL PAPER

The role of wax and resin in the nestmate recognition system of a stingless bee, *Tetragonisca angustula*

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Abstract Recent research has shown that entrance guards of the stingless bee *Tetragonisca angustula* make less errors in distinguishing nestmates from non-nestmates than all other bee species studied to date, but how they achieve this is unknown. We performed four experiments to investigate nestmate recognition by entrance guards in *T. angustula*. We first investigated the effect of colony odours on acceptance. Nestmates that acquired odour from non-nestmate workers were 63% more likely to be rejected while the acceptance rate of non-nestmates treated with nestmate odour increased by only 7%. We further hypothesised that guards standing on the wax entrance tube might use the tube as an odour referent. However, our findings showed that there was no difference in the acceptance of non-nestmates by guards standing on their own colony's entrance tube versus the non-nestmate's entrance tube. Moreover, treatment of bees with nestmate and non-nestmate resin or wax had a negative effect on acceptance rates of up to 65%, regardless of the origin of the wax or resin. The role of resin as a source of recognition cues was further investigated by unidirectionally transferring resin stores between colonies. Acceptance rates of nestmates declined by 37% for hives that donated resin, contrasting with resin donor hives where acceptance of non-nestmates increased by 21%. Overall, our results confirm the accuracy of nestmate recognition in *T. angustula* and reject the hypothesis that this high level of accuracy is due to the use of the wax entrance tubes as a referent for colony odour. Our findings also suggest that odours directly acquired from resin serve no primary function as nestmate recognition cues.

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The lack of consistency among colonies plus the complex results of the third and fourth experiments highlight the need for further research on the role of nest materials and cuticular profiles in understanding nestmate recognition in *T. angustula*.

Keywords Jataí · Meliponini · Stingless bees · Recognition template · Nestmate recognition

Introduction

Recognition of self versus non-self is ubiquitous among organisms, operating at several different levels and involving a variety of mechanisms (e.g. Beale 1990; Janeway and Medzhitov 2002; Nasrallah 2002; Glass and Kaneko 2003). Eusocial insects demonstrate self versus non-self recognition predominantly at the colony level (but see Tibbetts 2002; d'Ettorre and Heinze 2005). In many species, the nest entrance is defended by guards who deter both allospecific and conspecific intruders (Butler and Free 1952; Bell et al. 1974; Wittmann et al. 1990). Conspecific recognition requires the matching of a set of cues carried on the cuticle of an encountered individual (the label) with a previously acquired representation of colony odour (the template) of an evaluating individual (van Zweden and d'Ettorre 2010). Depending on the degree of similarity/dissimilarity, the encountered conspecific is accepted or rejected (Lacy and Sherman 1983; Vander Meer et al. 1998). Ideally, nestmate recognition should categorise all incoming individuals without error (Sherman et al. 1997), but mistakes are made: nestmates may be rejected (rejection errors) or non-nestmates admitted (acceptance errors). Which of these two errors is minimised can vary adaptively via adjustment of the acceptance threshold. For example, increased rejection errors may be traded off for decreased acceptance errors when the frequency of intruders or the cost of admitting them is higher (Reeve 1989; Downs and Ratnieks 2000; Couvillon et al. 2009).

In the honeybee, *Apis mellifera*, the number of entrance guards and the permissiveness of the acceptance threshold change adaptively, depending on nectar availability and robbing intensity (Downs and Ratnieks 2000; Couvillon et al. 2008). Overall, the recognition error rates are surprisingly high, with means of approximately 23% (range 8–48%) for rejection errors and 29% (range 21–62%) for conspecific acceptance errors (Breed 1983; Downs and Ratnieks 1999, 2000; Couvillon et al. 2007a, 2008, 2009, 2010). This gives a total error of approximately 52%, almost exactly midway between the two extremes of perfect (0%) and zero information (100%) (Ratnieks 1991). This is in stark contrast to recent results for the Neotropical stingless bee, *Tetragonisca angustula*. Guards of *T. angustula* made few errors in discriminating nestmate workers from non-nestmate conspecifics, accepting all nestmate workers (0% rejection errors) while rejecting 92% of conspecific non-nestmate workers, giving a total error of only 8% (Kärcher and Ratnieks 2009). This is also considerably lower than the error rates reported for five other Neotropical stingless bees (Table 1).

This raises the question on what the underlying mechanisms are that allow *T. angustula* to have lower recognition error rates than honeybees or other stingless bees. One obvious difference between *T. angustula* and the six other bee species is that *T. angustula* is the only one that constructs a wax entrance tubes for its nest. Nests of *Frieseomelitta varia* have a round entrance hole surrounded by resin, nests of *Trigona fulviventris* have wide, sometimes tubular, resin openings, while those of the three *Melipona* species, *Melipona quadrifasciata*, *Melipona rufiventris* and *Melipona scutellaris*, all possess a small entrance hole surrounded by dry mud (Roubik 2006; Couvillon et al. 2007b; M.J. Couvillon, personal communication; S.M. Jones, personal observation). Wax is important in honey bee recognition, functioning as the primary source of colony odour cues and a wax entrance tube might provide guards with a more direct template with which to compare incoming bees (Breed et al. 2004; Couvillon et al. 2007a). This might allow guards to update their template more frequently to allow peripheral sensory detection via desensitisation (c.f. Ozaki et al. 2005) or to simply enable a direct comparison.

A further difference between *T. angustula* and *A. mellifera* is the former's greater use of plant resins. Leonhardt et al. (2009) recently demonstrated that terpenoid profiles, derived from resin, extracted from the cuticles of seven Paleotropical stingless bee species varied quantitatively between colonies of the same species, leading them to suggest that this may potentially serve some communicative function in these stingless bee species. This is entirely feasible given that conspecific recognition may rely on quantitative differences within the same set of compounds (vander Meer et al. 1989; Espelie et al. 1990; Martin et al. 2008; van Zweden and d'Ettorre 2010). Nests of *T. angustula* contain substantial amounts of resin stored in numerous piles throughout the nest. Under a microscope (magnification × 240), resin can also be seen in a layer on the legs, head and thorax of foragers (J.S. van Zweden, unpublished data) and is also mixed with wax to form cerumen, which is used to construct the combs and surrounding involucrum (Nogueira-Neto 1997; S.M. Jones, personal observation; Wille 1983). Thus, the ubiquitous presence of resin within the nest, either in its pure form as piles or as cerumen, should be sufficient for acquisition of a colony-encompassing odour profile. Indeed this would in many ways be analogous to the ubiquitous presence of wax in the combs of honeybees, although wax is secreted by the

Table 1 Error rates for *T. angustula* and six other bee species

Bee species	Rejection error rate (%)	Acceptance error rate (%)	Total error rate (%)	Reference(s)
Apis mellifera	33	31	64	Breed 1983
	26	18–30	44–56	Couvillon et al. 2007a
	26–48	30–59	56–107	Couvillon et al. 2008
	19–24	57–62	76–86	Couvillon et al. 2009
	8	30	38	Couvillon et al. 2010
	18	21	39	Downs and Ratnieks 1999
	17	22	39	Downs and Ratnieks 2000
Frieseomelitta varia	11	27	38	Couvillon and Ratnieks 2008
Melipona quadrifasciata	0	26	26	Breed and Page 1991
Melipona rufiventris	0	86	86	Breed and Page 1991
Melipona scutellaris	0	40	40	Breed and Page 1991
Tetragonisca angustula	0	8	8	Kärcher and Ratnieks 2009
Trigona fulviventris	24	24	48	Buchwald and Breed 2005

bees while resin is collected (Breed et al. 1995; d'Ettorre et al. 2006).

The aim of this study was to investigate conspecific recognition in *T. angustula*, with emphasis on the effects of odours derived from wax entrance tubes, plant resins and worker bees. This was achieved by investigating whether the acceptance of introduced nestmates and non-nestmates by guards standing on the entrance tube was influenced by: (1) the acquisition of cuticular odours derived from nestmates and non-nestmates onto the cuticle, (2) swapping wax entrance tubes between colonies, (3) the acquisition of resin and wax derived from nestmates and non-nestmates onto the cuticle and (4) the unidirectional swap of entire resin stores between hives.

Methods

Study site and organism

Data were collected in February 2009 (experiments 1 and 2) and 2010 (experiments 3 and 4) at Fazenda Aretuzina, São Simão, São Paulo State, Brazil. During both study periods, the weather was hot, with daytime high temperatures of ca. 24–32°C and periodic heavy rain. Data were only collected on non-rainy days during active foraging (between 9.00 and 17.00 hours).

T. angustula, local name Jataí, is unique among the stingless bees in possessing two types of guards: both hovering and standing (van Zweden et al. 2011). Hovering guards flank the flight path leading to the nest and readily attack allospecific bees approaching the nest vicinity (Wittmann 1985; Grüter et al. 2011), thus increasing the defensive perimeter of the nest (van Zweden et al. 2011). While hovering guards are efficient at detecting individuals visually dissimilar to themselves, it is the role of the standing guards, which stand around the opening on the tip of the entrance tube, to distinguish non-nestmate conspecifics from nestmates, which they do by contact chemoreception (Kärcher and Ratnieks 2009). The two types of guards complement one another and increase the defensive efficiency of the colony.

We studied five colonies of *T. angustula* in 2009 (experiments 1 and 2) and six in 2010 (experiments 3 and 4). Each colony was housed in a wooden hive box (ca. 50-cm high× 20 cm×30 cm), with a circular entrance hole of 1.8 cm in diameter (for more details, see Nogueira-Neto (1997)). Each colony had built a wax entrance tube from this hole. Entrance tubes were ca. 1–3 cm long and had a circular opening at the tip ca. 0.6 cm in diameter (see also figures in Wittmann (1985); Couvillon et al. 2007b; for more detail, see Grüter et al. (2011)). The entrance tubes on the study colonies appeared identical to those of unmanaged colonies nesting in walls. Hives were raised ca. 1 m aboveground on hive stands or attached to the walls of buildings. The study colonies were queenright and thriving, with the hive nearly full of combs, covered by involucrum and numerous honey pots with a population of many thousands of workers. Mature colonies of *T. angustula* in Costa Rica were estimated to have approximately 10,000 workers (van Veen and Sommeijer 2000).

Introduction of worker bees to guards and their acceptance or rejection

The acceptance or rejection of conspecific workers by guards standing on the entrance tube was determined using a standard bioassay (Downs and Ratnieks 2000) developed for studying honey bees, *A. mellifera*, and modified for use with *T. angustula* (Kärcher and Ratnieks 2009). Returning

foragers were collected at the hive entrance, placed in a tube and immediately chilled in an ice chest for 10–20 min, then removed one at a time and allowed to warm to ambient temperature. Once warmed, these workers walked actively but were less likely to fly than previously unchilled workers. A worker was taken from the ice chest and, once warmed up, allowed to grasp a clean wooden toothpick and walk onto the outer surface of the tip of the entrance tube of a discriminator hive. On contact with the guards standing on the entrance tube behaviour was observed for up to 2 min. The introduced worker was considered "rejected" if it was bitten and tugged for the duration of the observation period or fell off the tube while grappling with a guard (Kärcher and Ratnieks 2009). The worker was considered "accepted'" if she was subjected only to licking and antennal contact or bitten and tugged for a few seconds and then left alone. Each time four bees were introduced in a row to a particular discriminator hive pseudorandomly with approximately 5 min between each introduction. Once the four bees had been introduced, the same protocol was repeated at the next discriminator hive, ensuring that a minimum of 45 min had passed before returning to a particular discriminator hive. The observer was unaware of the treatment group of the introduced workers. The number of standing guards present on the entrance tube was recorded before introductions commenced (mean ± SD=15.43±4.38).

Experiment 1: the effect of bee-derived odours on acceptance rates of worker bees

The aim of this experiment was to determine how the transfer of nestmate and non-nestmate odours onto worker bees affected the acceptance of both nestmates and non-nestmates.

Four hives (A–D) were used both as discriminator and donor colonies. These were grouped into two pairs (A and B, C and D) to serve as donors and discriminators to each other (Fig. 1a). A fifth hive (E) was used as an additional source of non-nestmates. Worker bees (n=20±3) were collected at hive entrances and placed in a 6-ml clear plastic vial for 60 min to transfer odours to the vial at two vials per hive per study day. The bees were then released. Odours deposited on the inside of the tubes by these bees were then indirectly transferred to returning foragers by placing 12 individuals into a prepared vial for 15 min. Each vial was used only once. Fresh vials were prepared on each study day and used within 4 h.

The acceptance rate of the following seven treatments of workers were compared (Fig. 1a): (1) nestmates, untreated; (2) nestmates, treated with nestmate odour using the vial; (3) nestmates, treated with non-nestmate odour from the paired hive using the vial; (4) non-nestmates from the paired hive, untreated; (5) non-nestmates from hive E, untreated; (6) non-nestmates, treated with non-nestmate odour from the paired hive; and (7) non-nestmates, treated with nestmate odour.

Combined sample sizes for each of the seven treatments ranged between 99 and 111, with similar numbers introduced to each of the four discriminator hives.

Experiment 2: is the wax entrance tube used as a referent?

The aim of this experiment was to determine whether *T. angustula* guards use the wax entrance tube as a template or referent for colony odour. To achieve this, we swapped entrance tubes between paired colonies using the same pairings as in experiment 1. The entrance tube was gently cut away from the hive entrance hole using a penknife. By using the natural stickiness of the wax, the entrance tube could be attached to the end of a 1.5-cm-long plastic tube that exactly fitted into the hive entrance hole. The plastic tube was then placed into the hive's entrance hole and the colony was given 1–3 days for the entrance tube to attach firmly to the plastic tube using additional wax. Entrance tubes could then be swapped between hives in minutes, without physical damage and with minimal disturbance. Following tube swapping, guards appeared to behave normally on the new entrance tube.

The experimental design was the same as that used for experiment 1, with the exception that acceptance rates were determined only for the following treatments: (1) nestmates, (2) paired hive non-nestmates and (3) hive E non-nestmates. These were compared before and 1, 5 and 24 h after the swap (Fig. 1a).

Combined sample sizes ranged between 101 and 120 introduced bees for each of the three treatments both before and after the tube swap. Similar numbers were introduced to each of the four discriminator hives for each treatment.

Experiment 3: transfer of wax and resin odours to workers

The aim of this experiment was to determine if resin- or wax-derived recognition cues were utilised by *T. angustula* for the purpose of nestmate recognition. Six hives (F–K) were used: three discriminator colonies and three donor colonies for wax, resin and non-nestmates (Fig. 1b). Each discriminator colony thus received workers from all of the donor colonies in addition to their own nestmates. This change to the design from that used in experiments 1 and 2 diminishes any possible colony-specific effects as these can be dealt with statistically. Each discriminator hive received bees from its own hive and each of the three donor hives (i.e. a full factorial design) were treated as follows: (1) nestmates untreated, (2) nestmates treated with nestmate resin/wax, (3) nestmates treated with non-nestmate resin/wax, (4) non-

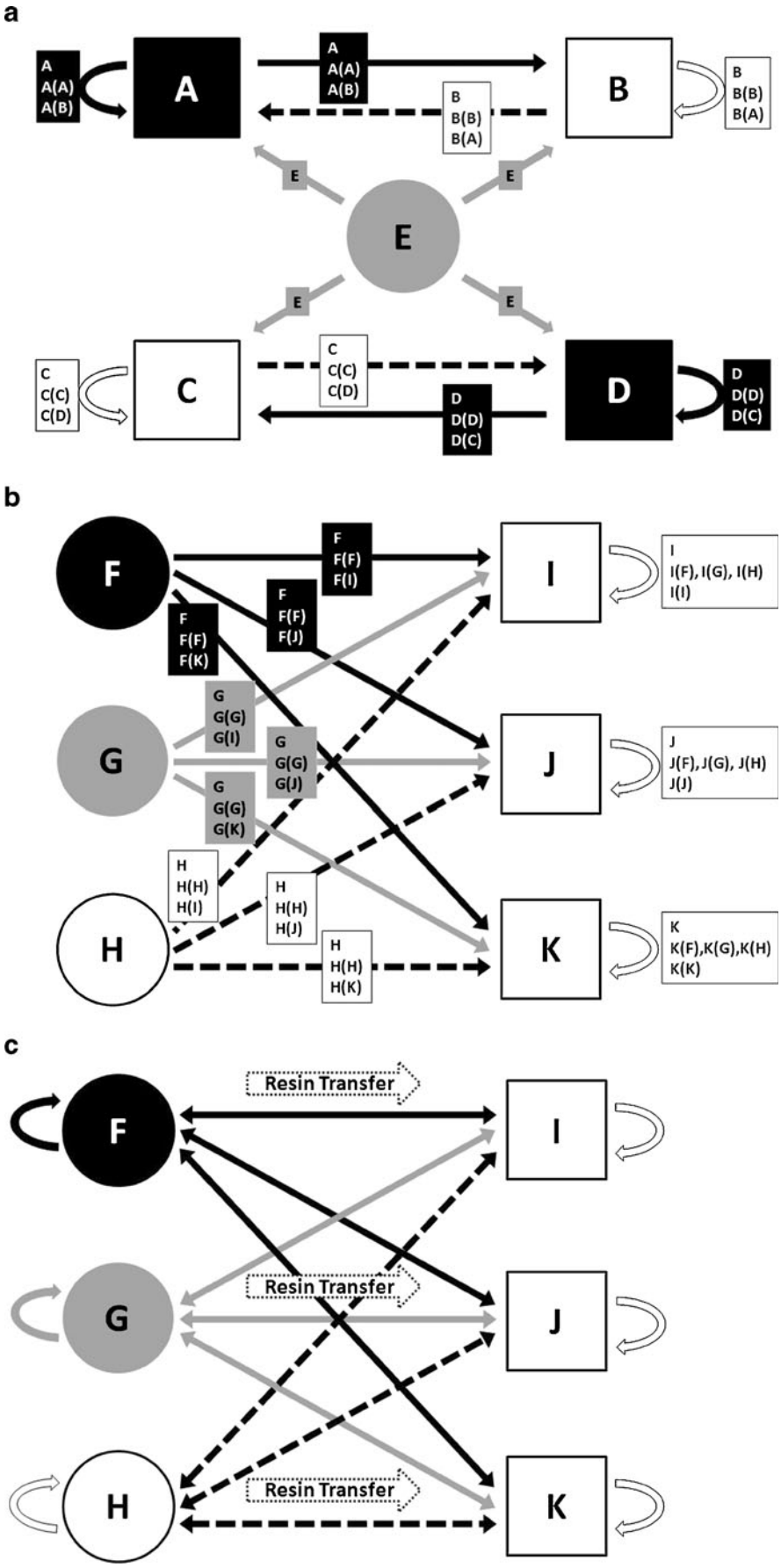

Fig. 1 **a** Experimental design of experiments 1 and 2. Treated and untreated worker bees were introduced to the entrance tubes of each discriminator hive. There were two pairs of discriminator hives (one black and one white). Hive E served as an additional source of non-nestmates, common to all four colonies. In the *boxes on the arrow*, *unbracketed capital letters* refer to the colony the introduced bee is from and the *bracketed letters* refer to the colony that the odour treatment, if any, originates from. **b** Experimental design of experiment 3. Treated and untreated worker bees were introduced to the three discriminator hives, *I*, *J* and *K*, from the donor hives, *F*, *G* and *H*. **c** Experimental design of experiment 4. Untreated worker bees were introduced to the entrance tubes of six discriminator hives *F–K*. Hives *F–H* acted as "resin donors" and hives *I–K* as "resin recipients". Resin was swapped unidirectionally from the donor hives to the recipient hives. Worker bees were subsequently introduced to all hives 24 h afterwards for a period of 7 days

nestmates untreated, (5) Non-nestmates treated with nestmate resin/wax and (6) non-nestmates treated with non-nestmate resin/wax. Combined sample sizes per treatment ranged between 35 and 102 introduced bees with similar numbers introduced to the three discriminator hives.

Resin was collected from resin piles within each colony and white wax was collected from newly constructed entrance tubes. We are confident that the resin we collected from the piles contained little or no wax because its dark colour and viscous consistency was identical to the resin carried in the corbiculae of returning foragers. Moreover, Gastauer et al. (2011) observed no mixing of wax or other substances with the resin collected by worker bees of seven Neotropical stingless bee species, including *T. angustula*. Each 4-ml glass vial was treated with 0.5 ml of hexane containing 2.5 mg of either wax or resin. Evaporation left a thin, barely visible coating within each vial. Up to four workers were transferred to a treated vial and left for at least 15 min to allow indirect transfer. The bees were then chilled and introduced individually to the entrance tube of a discriminator colony as in experiment 1. Each vial was used up to three times to treat a maximum of ten bees.

Experiment 4: one-way transfer of resin between hives

The aim of this experiment was to investigate the effect on the nestmate recognition label and/or template of unidirectional transfer of resin between *T. angustula* hives. Hive inspections showed that all the *T. angustula* nests had resin piles of varying sizes, all dark brown in colour, which were distributed throughout the nest. The mean weight of the entire resin reserves for the six colonies was 7.79±2.01 g (mean±1 s.e., range=2.04–16.05 g).

Entire resin stores were removed from a donor hive, weighed and distributed as new piles within a receiving hive that had been cleared of existing resin piles the day before. Six colonies were used (F–K), paired up as three groups containing a ‘resin donor’ and ‘resin acceptor’ (F and I, G and J, H and K; Fig. 1c). Bees were introduced to all hives prior to and following the swap. Each donor hive received nestmates and non-nestmates from each of the three resin acceptor hives and vice versa for the receiving hives. Depending on the treatment, combined sample sizes ranged between 17 and 126 introduced bees. Introductions were undertaken at four different time periods: between 12 and 96 h before the resin transfer (control) and then at 12, 60 and 84 h after.

Statistical analyses

For data analysis, we used generalised linear mixed-effect models (GLMM) with binomial errors in R 2.9 (R Development Core Team 2009). We fitted the models using the lmer function (Bates 2007). Colony was included as a random effect throughout to control for the non-independence of data points from the same colony (Bolker et al. 2009; Zuur et al. 2009). For model selection, we used the protocol proposed by Zuur et al. (2009). We first explored the optimal structure of the random components by comparing random intercept models with random intercept and slope models. Then, we explored the optimal fixed component structure. Wald tests were used to determine the significance of the fixed effects (Bolker et al. 2009).

For all cases, the dependent variable was the response of the guards (accept or reject). The random variable was “discriminator colony” in all experiments. Fixed variables were “treatment” in experiment 1, “time (time following entrance tube swap)” in experiment 2, “treatment” and “origin” of bee (nestmate or non-nestmate) for experiment 3 and “treatment”, “time” (before or after swap) and “origin” of bee for experiment 4.

Results

Experiment 1: the effect of odour transfer on acceptance rates of worker bees

Guards standing on the entrance tubes made few recognition errors with untreated introduced bees, accepting significantly more nestmates than non-nestmates as expected (Fig. 2; 94.6% vs. 4.5%, GLMM, Wald’s $z=-14.15$, $p=<0.001$). The strongest effect of odour treatment came from treating nestmates with non-nestmate odour, which resulted in an acceptance 54.3% lower than for nestmates treated with nestmate odour (31.3% vs. 85.6%, $z=-7.41$, $p=<0.001$). Conversely, the acceptance rate of non-nestmates was not significantly affected by treatment; only 6.8% more non-nestmates were accepted when treated with nestmate odour than when treated with non-nestmate odour (9.6% vs. 2.8%, $z=1.92$, $p=0.054$). A small but significant effect of the vial treatment itself could be seen on acceptance rates of bees treated with nestmate odour (94.6% vs. 85.6%, $z=-2.03$, $p=0.04$).

Experiment 2: is the wax entrance tube used as a referent?

Swapping entrance tubes did not affect the acceptance of either nestmates or non-nestmates (Fig. 2). There was no significant difference between the acceptance rates of nestmates for the four different time periods individually (0 h vs. 1 h: $z=-0.46$, $p=0.65$; 0 h vs. 5 h: $z=-1.83$, $p=0.067$; 0 h vs. 24 h: $z=0.14$, $p=0.91$) and combined (0 h vs. 1/5/24 h: 94.6% vs. 90.8%, $z=-1.05$, $p=0.29$). Similarly, there was no significant difference among the acceptance

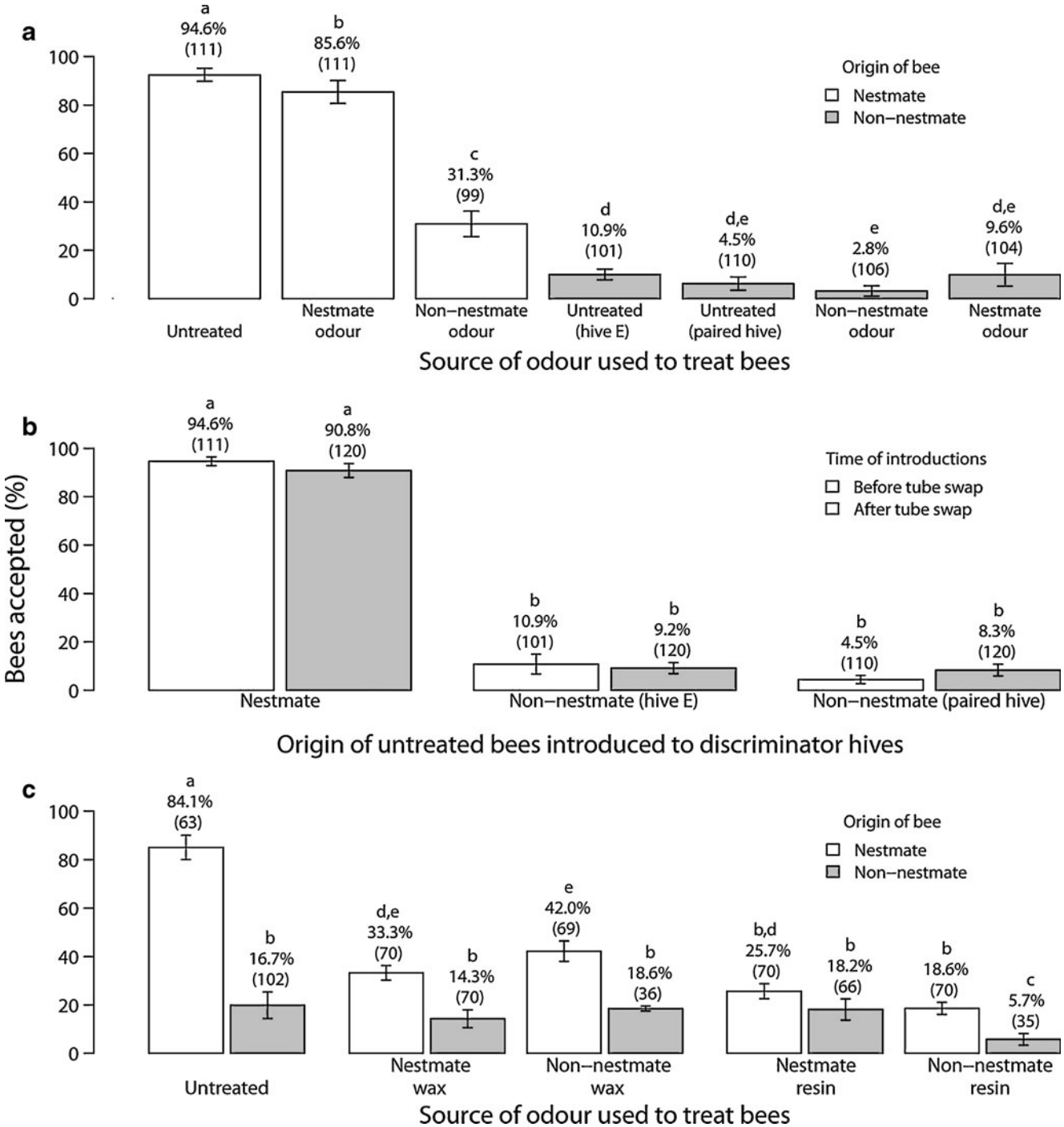

Fig. 2 Acceptance rates of introduced bees in experiments 1, 2 and 3. **a** Experiment 1: Treated and untreated worker bees were introduced to four discriminator hives, A, B, C and D. Non-nestmates introduced to discriminator hives originated from the paired hive and a fifth colony, colony E (untreated only), which served as a control. **b** Experiment 2: Nestmate workers and non-nestmate workers originating from both the paired hive and hive E (control) were introduced to four discriminator hives, both before and after swapping the wax entrance tubes. **c** Experiment 3: Nestmates and non-nestmate workers, either untreated or treated, were introduced to three discriminator hives. Treated bees bore wax- or resin-derived odours from either their own hive or a foreign hive. *Different letters* denote significant differences. Exact percentage acceptance rates and sample sizes are given above the *bars*

rates of paired hive non-nestmates for the different time periods, both individually (0 h vs. 1 h: z=0.79, p=0.43; 0 h vs. 5 h: z=0.79, p=0.43; 0 h vs. 24 h: z=1.32, p=0.19) and combined (0 h vs. 1/5/24 h: 4.5% vs. 8.3%, z=1.27, p=0.21). As expected, there was also no change in the acceptance of non-nestmates from hive E post-swap (0 h vs. 1/5/24 h: 10.9% vs. 9.2%, z=1.26, p=0.21). In addition, there is no indication that tube swapping affected the

acceptance of non-nestmates from the paired colony any differently than non-nestmates from the control, hive E, with no significant interaction between treatment and tube swapping (pre-swap vs. post-swap; $z=1.27$, $p=0.21$).

Experiment 3: transfer of wax and resin odours to workers

Treatment of nestmate bees with either wax or resin lowered their acceptance rates significantly to that of non-nestmates, irrespective of whether the wax/resin originated from a nestmate or non-nestmate hive (Fig. 2; 84.1% vs. 33.3%, $z=4.78$, $p=<0.001$; 84.1% vs. 42.0%, $z=4.71$, $p=<0.001$; 84.1% vs. 25.7%, $z=5.27$, $p=<0.001$; 84.1% vs. 18.6%, $z=6.81$, $p=<0.001$). Acceptance of non-nestmates remained low regardless of treatment (16.7% vs. 14.3%, $z=0.41$, $p=0.67$; 16.7% vs. 18.6%, $z=-0.33$, $p=0.73$; 16.7% vs. 18.2%, $z=-0.26$, $p=0.79$; 16.7% vs. 5.7%, $z=2.05$, $p=0.040$). Interestingly, there were no pronounced differences between resin and wax sourced from nestmate and non-nestmate hives. The acceptance rates of nestmates treated with nestmate or non-nestmate wax did not differ significantly (33.3% vs. 42.0%, $z=-0.86$, $p=0.39$), reflecting what we found for non-nestmates with the same treatments (14.3% vs. 18.6%, $z=-0.68$, $p=0.49$). Similarly, the acceptance rates of nestmates treated with nestmate or non-nestmate resin did not differ significantly (18.6% vs. 25.7%, $z=0.84$, $p=0.39$). However, non-nestmates treated with non-nestmate resin were rejected to a greater extent than nestmates treated with non-nestmate resin (5.7% vs. 18.2%, $z=2.14$, $p=0.03$).

Experiment 4: one-way transfer of resin between hives

After the unidirectional transfer of resin, the acceptance rate of nestmates dropped by 37.5% for resin donor hives (from 81.9% to 44.4%, $z=-3.51$, $p=<0.001$; Fig. 3), while only a decline of 4.5% was seen for resin recipient hives (from 82.3% to 77.8%, $z=-0.36$, $p=0.71$). Conversely, for non-nestmates, a non-significant increase of 1.6% in acceptance rates was seen in donor hives (from 14.3% to 15.9%, $z=0.35$, $p=0.72$), while a significant rise of 21.6% was observed for recipient hives (from 6.2% to 27.8%, $z=2.75$, $p=0.005$). This effect was independent of the resin source, that is, acceptance rates did not differ between non-nestmates from the paired hive and non-nestmates from other hives ($z=-1.03$, $p=0.30$). The overall trends were somewhat inconsistent amongst the hives with notable variation apparent (see "Electronic supplementary material").

Discussion

The results of the first experiment involving transferral of bee-derived odours confirm the exceptional recognition abilities of *T. angustula* compared to other studied species, with combined recognition errors of 10%. Acceptance rates of nestmates treated with non-nestmate odour were greatly and significantly reduced, while the small positive effect on acceptance from treating non-nestmates with nestmate odour was non-significant. Together with recent studies on the honeybee (Ratnieks et al. 2011), the stingless bee *F. varia* (Couvillon and Ratnieks 2008) and *Camponotus* ants (Guerrieri et al. 2009), these results support the idea that the odour space of a particular colony odour utilised in nestmate recognition is complex and multidimensional. In particular, a multidimensional odour space helps explain why it is far easier for a nestmate odour to diverge from the colony odour rather than a non-nestmate odour to converge on the colony odour of the guards (Ratnieks et al. 2011). In this framework, the average chemical distance that an individual is moved following odour transfer is enough to make a nestmate unacceptable but not enough to make a non-nestmate acceptable even though both changes are equal in chemical distance. Nestmate odours are chemically similar and therefore fall within the overall colony odour profile. Any deviation therefore is easily recognised; however, non-nestmate odours are sufficiently distant in chemical space that the same deviation, even towards the nestmate odour profile, is not enough to fall within the colony odour profile (for a visual representation, see Fig. 3 in Ratnieks et al. 2011).

Swapping of entrance tubes had no effect on acceptance and allows us to reject the hypothesis that accurate nestmate recognition in *T. angustula* is due to the wax entrance tube serving as a convenient or immediate template for colony odour. Our data show a small but non-significant 3% increase in the acceptance rates of non-nestmates introduced after tube swapping, which is only 1/30th of the 90% difference seen between the acceptance of untreated nestmates (94.6%) and untreated non-nestmates (4.5%) observed in our first experiment. Our results also show no significant change in acceptance rates with time, from 1 to 24 h post-tube swap. We would expect to see an effect in this time frame as swapping of wax combs in honeybees leads to a change in behaviour within hours (Couvillon et al. 2007a).

While the first two experiments both provided very clear findings, the results of the subsequent two were less clear. Following treatment of worker bees with wax and resin odours, we found no difference between acceptance rates of bees treated with nestmate wax or resin versus non-nestmate wax or resin. If wax or resin serves as a source of colony odour, we would have expected to see a disparity between nestmates treated with their own wax or resin versus non-nestmate wax or resin, as seen in the first experiment where the odours in the vial were derived from live bees. Acceptance rates of nestmates dramatically

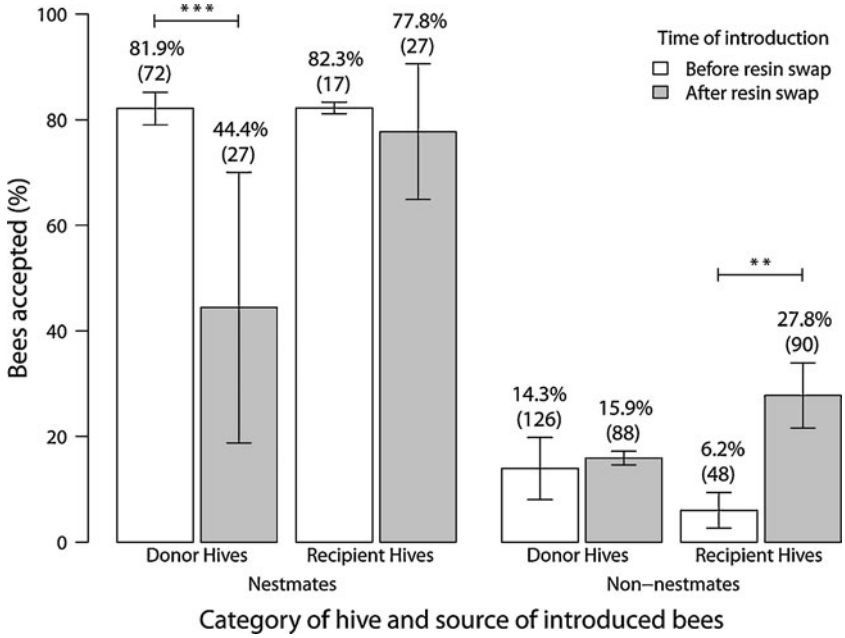

Fig. 3 Experiment 4: Nestmate and non-nestmate workers were introduced to six discriminator hives, both before and after a unidirectional resin transfer. Three discriminator hives were resin donors and three were resin recipients, forming three pairs. Exact percentage acceptance rates and sample sizes are given above the *bars*. Statistically significant differences are indicated (**$p<0.01$, ***$p<0.001$)

dropped instead (by 50.8% and 42.1% for wax and by 58.4% and 65.5% for resin) regardless of whether the wax or resin originated from a nestmate or a non-nestmate hive, respectively.

Transfer of resin stores between hives was our second approach to investigate the possible role of resin in nestmate recognition. In hives that had donated resin, acceptance rates of non-nestmates remained the same following resin transfer while acceptance rates of nestmates declined by over 37% (Fig. 3). Conversely, hives that had received resin accepted a significantly greater number of non-nestmates (an increase of 21%), while the acceptance rate of nestmates remained the same. We had expected to see a trade-off in which an increase in acceptance errors and a simultaneous decrease in rejection errors both occurred (Reeve 1989; Couvillon et al. 2009), and vice versa, but this negative correlation was not observed. If the template of the guards had been updated following the introduction of resin, then we would have expected to see a rise in the acceptance rate of non-nestmates from the partnered donor hive (c.f. Couvillon et al. 2007a). Although this effect is apparent, the acceptance rate of non-nestmates from non-partnered hives also increases to the same degree. At face value, it appears that guards were unable to distinguish between non-nestmates introduced from their partnered hive versus other non-nestmate hives. However, the interpretation of these trends is complicated by the fact that there was great variation in acceptance rates between the six discriminator hives (see "Electronic supplementary material"). For example, acceptance rates of nestmates by resin donor hives varied from 0% to 89% following the resin transfer. This marked variation suggests that something else may be occurring which our experiment was unable to reveal and therefore warrants further investigation.

The behaviour shown by the guards of resin donor hives is also puzzling. The post-transfer decline in acceptance of nestmates may be a response to the loss of the colony's entire resin store, but if this were the case we would predict a simultaneous increase in rejection rates of non-nestmates, which was not seen. The high variation in acceptance rates evident within both the donor and recipient colonies is perhaps indicative of guard confusion. Indeed this was conspicuous with guards exhibiting frequent and intense antennation with greater periods of time preceding rejection (S.M. Jones, personal observation). This lack of consistency in changes in acceptance rates among the discriminator colonies was also apparent in the wax and resin odour transfer experiment and is notably different from the consistent changes seen in the first two experiments. Our findings appear to show that *T. angustula* do not use pure resin as a source of cues for nestmate recognition. Several studies have failed to identify the presence of terpenoids on the wings of various Neotropical stingless bees (Abdalla et al. 2003; Jungnickel et al. 2004; Kerr et al. 2004; Nunes et al. 2008). To our knowledge, no study has yet analysed the cuticular chemical profiles of *T. angustula* but it would be surprising if terpenoids were absent when we know that resin, which is a rich source of terpenoids (Velikova et al. 2000; Sawaya et al. 2006), is found on the thorax of many foragers (J.S. van Zweden, unpublished data) and is universally present within the hive both in resin piles or mixed with wax as cerumen (S.M. Jones, personal observation; Michener 1974).

It is possible that the inconsistent results seen for the resin transfer experiment may have arisen because the

terpenoid composition of the pure resin we collected from the resin piles does not reflect the terpenoid profile present on the cuticles of the bees. Leonhardt et al. (2011) showed that terpenoids present on the cuticles of six different Paleotropical stingless bees differed from those found on nest material. Leonhardt et al. (2011) were also able to show that, from a total of 1,117 terpenoids available in stored resin, only 10% (105) were actually present on the cuticle of the Paleotropical stingless bee, *Tetragonilla collina*. To explain this, Leonhardt et al. (2011) proposed a hypothesis whereby stingless bees are able to perform some form of post-collection manipulation of resin terpenoids to ensure odour constancy. Resin stored by colonies of *T. angustula* from across Brazil was found to have a remarkably consistent composition, regardless of location (Sawaya et al. 2006). Therefore, for terpenoids to function as cues for nestmate recognition, quantitative differences between a discrete set of these compounds must be apparent among colonies and this would have to be achieved by some form of post-collection manipulation. Confirmation of whether terpenoids can be manipulated in this manner or indeed function as suitable recognition cues will require further behavioural and analytical study. A more parsimonious explanation may be that resin simply does not function as a primary source of recognition cues in *T. angustula*. An inherent problem with using collected materials, such as resin or food, as odour cues is the likelihood that the availability of the sources will change with time (Downs et al. 2000, 2001). Once a bee collects new material which is not consistent with its colony odour, there is a strong possibility that it will be rejected. For example, floral odours, most of which are terpenoids, were found to have no function in honey bee nestmate recognition (Downs et al. 2000).

Overall, our results confirm the accuracy of the nestmate recognition system in *T. angustula*. When results of the controls from the first three experiments were combined, a typical average of 10% was observed for both acceptance and rejection errors, giving a total error rate of 20%. Despite the variation that exists between colonies and studies, the error rate remains considerably lower than those reported for honey bees (Downs and Ratnieks 1999, 2000; Couvillon et al. 2007a, 2008, 2009, 2010) and lower than all stingless bee species studied to date (Breed and Page 1991; Buchwald and Breed 2005; Couvillon and Ratnieks 2008). Although our results do not show how *T. angustula* achieves this accuracy, we have ruled out one strong contender: the wax entrance tubes of *T. angustula* nests appear to play no role in nestmate recognition. Our results from the resin odour treatment and resin transfer experiments suggest that odours acquired directly from resin also serve no function as nestmate recognition cues, although the observed shifts in the acceptance threshold seen for the resin transfer suggest a possible secondary role. However, the variation and inconsistency of our results in the last two experiments together highlight the need for future chemical analysis of resin stores, cerumen and the cuticular profiles of worker bees. Indeed the results of a recent study by Nunes et al. (2011) suggest that cerumen may be a source of recognition cues, used by colony members of the stingless bee *F. varia*. It also remains to be seen whether this proficient recognition system has evolved as a result of low genetic variability or high parasite pressure.

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