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ORIGINAL PAPER

Soldier production in a stingless bee depends on rearing location and nurse behaviour

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Abstract The spectacular morphological variation among workers of certain ant and termite species has fascinated evolutionary biologists since Darwin. In some species, environmental triggers induce larvae to develop into different phenotypes, e.g. minor or major workers (soldiers). Recently, the first soldier subcaste was discovered in a bee, the stingless bee *Tetragonisca angustula*. In contrast to ants, which raise their offspring by progressively feeding larvae until the pupal stage, *T. angustula* nurses mass provision individual brood cells after which the bees develop from egg to young workers in sealed cells on a seemingly uniform brood comb. This prompts the question of how this bee creates a morphologically variable workforce without larvae having direct contact with nursing workers. We investigated where *T. angustula* raises a larger soldier subcaste on its compact brood comb. Additionally, we examined whether size differences among workers could be generated by differential distribution of food by nursing workers. We found that colonies produce c. 1–6 % of soldier-sized workers, which mainly emerge from a small central area of the comb. In this area, cells are wider and a larger number of nursing bees unload larval food here before oviposition. Cell attendance levels prior to oviposition were similar across the comb and, thus, did not explain the larger food volumes found in the centre. Our results suggest that workers determine soldier production via larval food discharges and cell-building behaviour. Nutritional differences among larvae might then induce larvae into one or the other caste developmental pathway.

Keywords Morphological castes · Size polymorphism · Division of labour · Social insects · Stingless bees · ?Larval nutrition

Introduction

Worker differentiation within social insect colonies is a prime example of phenotypic plasticity. In response to environmental triggers, a differential expression of genes can give rise to individuals widely differing in morphology and behaviour (e.g. Smith et al. 2008; Rajakumar et al. 2012; Shpigler et al. 2013). Often, phenotypic differences among workers are linked to how a colony divides the labour, as tendencies to perform certain tasks are related to worker morphology (Oster and Wilson 1978; Hölldobler and Wilson 1990). Morphological (or physical) castes can be observed in several social insect taxa, such as termites (Noirot and Pasteels 1987), aphids (Stern and Foster 1996) and ants (Hölldobler and Wilson 1990).

Although ants provide some of the most striking examples of morphological castes (e.g. soldiers in *Atta* leafcutter ants, Hölldobler and Wilson 1990), this phenomenon is seen as relatively uncommon in eusocial Hymenoptera compared to

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its frequent occurrence in marine colonial invertebrates (Harvell 1994), and several explanations for the rarity of physical worker castes have been proposed: for example, morphological specialisation might reduce the reproductive potential of workers and, thus, individual-level selection might not favour it (Oster and Wilson 1978; Fjerdingstad and Crozier 2006). Furthermore, a relatively small queen-worker dimorphism is thought to set a limit to the evolution of morphological complexity by reducing the developmental flexibility of worker larvae (Wheeler 1986; Wheeler 1991; Fjerdingstad and Crozier 2006, for other hypotheses see e.g. Oster and Wilson 1978; Harvell 1994). In advanced eusocial bees (also called highly eusocial) in particular, worker size variation has long been thought to be too small to give rise to physical worker castes (Ramalho et al. 1998), and it has been viewed as a phenomenon with an unknown adaptive value (Waddington et al. 1986). However, recently, a morphological soldier caste in a stingless bee (*Tetragonisca angustula*) was discovered, with nest entrance guards (“soldiers”) being on average 30 % heavier than foragers and with proportionally different body parts (Grüter et al. 2012). Furthermore, soldiers show a long dedication to the guarding task (Grüter et al. 2011) and, importantly, size variation within task groups is small in *T. angustula*, leading to physically and functionally differentiated worker types. Among the stingless bees, the overall worker size variation found in *T. angustula* is one of the highest recorded so far (Waddington et al. 1986; Ramalho et al. 1998; Goulson et al. 2005; Quezada-Euán et al. 2011).

Like many stingless bee species, *T. angustula* builds compact horizontal combs which could inhibit large within colony size variation (Wille 1983; Roulston and Cane 2000; see Fig. S1 in Grüter et al. 2012). This is in contrast to ants, where larvae can develop without the constraint of a cell into widely different morphological castes (e.g. up to 200-fold difference in dry weight between *Atta sexdens* minors and majors, Hölldobler and Wilson 1990). Thus, the discovery of a soldier bee raises the question of how the size variation within the worker caste of *T. angustula* is generated. Does the soldier caste develop in larger brood cells? If so, where on the comb can such cells be found?

T. angustula colonies are, like in most stingless bee species, headed by a singly mated queen (Strassmann 2001). Thus, workers in a nest are full sisters with a high degree of relatedness. Therefore, size differences among nest workers are likely to be generated by environmental variation rather than genetic factors (e.g. Hughes et al. 2003; Evison and Hughes 2011). Generally, in insects, adult size is directly correlated with the quantity of food received as a larva (e.g. Shingleton et al. 2007; Radmacher and Strohm 2010; Quezada-Euán et al. 2011). Fluctuations in temperature and humidity can also affect growth in insect larvae (Emlen and Nijhout 2000), but because *T. angustula* envelops its brood combs in protective wax sheets (involucrum), temperature and humidity are likely to be highly similar for all brood and size differences are likely to reflect a differential energetic investment in larvae.

Since ant nurses feed the larvae progressively, the final size of an ant worker can be adjusted during the larval stage by varying the moment of metamorphosis and thus prolonging or shortening the period over which the larvae is fed (Wheeler 1991). In contrast, in stingless bees, the final size of a worker depends on the amount of food deposited by nurses in its cell before oviposition (Bego et al. 1999). After oviposition, cells are sealed immediately. Thus, if soldiers and minor workers require a differential energetic investment, variation in the distribution of food among stingless bee worker larvae has to exist without larvae being able to signal their needs to nurse workers. Here, we investigated if some brood cells of *T. angustula* receive more larval food from nursing workers and if so, how this is organised. We hypothesised that the size variation among *T. angustula* worker bees could be the result of a differential food distribution among cells by nursing workers. We examined this by counting the food discharges by nurses into cells and by measuring the food content of cells in different areas of the brood comb. Furthermore, we measured the size of workers emerging from different areas, and we tested if there are cells of different width on a *T. angustula* brood comb, as this could explain variation in the amount of discharges nurses give to cells.

Methods

Study species

T. angustula is a eusocial stingless bee species which is common in large parts of Latin America (Michener 2007). It builds colonies inside cavities in trees and buildings. Colonies have an unusual nest defence with two different types of guards. Hovering guards fly near the entrance and mainly detect allospecific intruders (Wittmann 1985; van Zweden et al. 2011). Standing guards stand on the wax-entrance tube (see Fig. 1 in van Zweden et al. 2011) and are mainly responsible for detecting conspecific intruders before they enter the hive (Jones et al. 2012; Couvillon et al. 2013). Both guard types show a long-term dedication to this task (Grüter et al. 2011) and are part of one morphologically distinct soldier caste which is on average 30 % heavier than foragers and has relatively longer hind legs and relatively smaller heads (n.b. absolutely guards have larger heads than foragers) (Grüter et al. 2012). *T. angustula* has the largest known queen-worker dimorphism among stingless bees (Tóth et al. 2004). Accordingly, *T. angustula* queen cells are visibly larger than worker cells, and none were present on the combs used in this study.

T. angustula constructs vertical brood cells organised in round, compact horizontal combs (Fig. S1 in Grüter et al. 2012), and new cells are built along the periphery of a comb. When the construction of a brood comb is nearly finished,

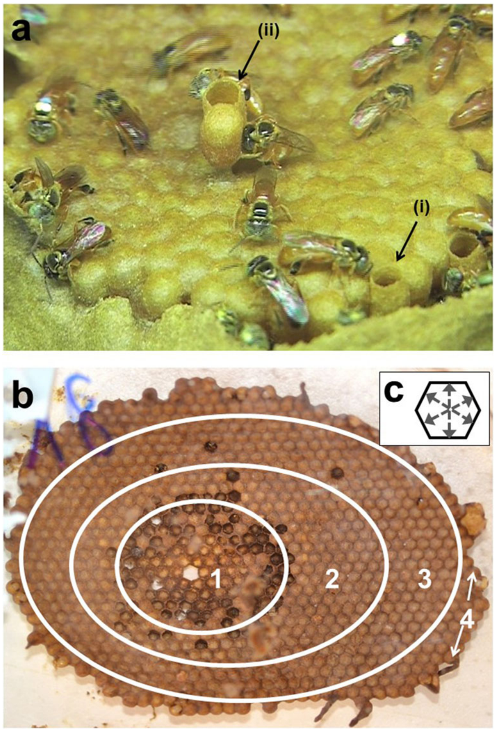

Fig. 1 **a** A typical brood comb of *T. angustula*. The *arrows* designate unfilled cells. Cell (*i*) is part of an older comb while (*ii*) indicates a centre cell of the new comb which is built on top. **b** The different brood comb areas used in the head width, cell width and cell content measurements. On this brood comb, workers of the centre (*1*) have already emerged. The next workers to emerge are workers of the outer centre (*2*). Later, workers of the periphery (*3*) will emerge before; finally, workers of the outer ring, i.e. the outermost row of cells, emerge (*4*). **c** Three measurements of cell width were taken for each cell and averaged (the three measurements are indicated by *arrows* inside the hexagonal cell)

workers start to build the next brood comb on top of it by making a thin wax pillar in the middle to which the first cells of the new comb are attached (Fig. 1a). Cells that are ready to be filled are easily recognisable by having an upward rim around the top ("collar"). The behaviour of *T. angustula* nurses during the provisioning and oviposition process (POP) is well described (Koedam et al. 1997; Bego et al. 1999): the presence of the queen stimulates the workers to start regurgitating larval food in collared cells. Larval food in stingless bees consists of a mixture of pollen, gland secretions and nectar in stable proportions at a given time of year (Quezada-Euán et al. 2011). The first discharge is soon followed by subsequent discharges by other individuals. Multiple cells can be provisioned with larval food simultaneously and the queen mainly lays eggs in batches. Often after the food provisioning of a cell has ended, a worker lays a trophic egg on the collar of the cell. When the queen arrives, she eats the trophic egg and afterwards places her abdomen in the cell to oviposit on top of the larval food. Immediately after the queen leaves, the workers seal the cell.

The colonies used in this study were kept on the Ribeirão Preto campus of the University of São Paulo. The head width, cell width and food weight measurements were done in February 2012, and the video recordings took place in May 2013, September 2013 and May 2014.

Measuring cell width

We collected six fully finished brood combs containing bees close to eclosion from six colonies (one per colony) and photographed the centre and the periphery (areas 1 and 3 in Fig. 1b) of each comb. We focused on these two areas because we hypothesised that cells in these areas might be most different in size. Small squares of graph paper positioned on top of the comb were used for size calibrations. With ImageJ software (1.46r), we took three measurements of cell width per cell (see Fig. 1c) and averaged them to obtain one value for each cell. In total, we measured 69 cells in the centre (7–21 per colony) and 76 cells in the periphery (8–24 per colony).

Measuring head width

After the cell size measurements, five of the six combs were placed in petri dishes (Corning 150×25 mm). One comb was discarded for further measurements as it was damaged while handling. Subsequently, we kept the combs in an incubator at a temperature of 28 °C. As *T. angustula* workers first build and provision the cells in the centre of a brood comb, this is the location from which the first young bees start to emerge. This way of brood rearing of *T. angustula* allowed us to collect successively workers that were reared on different areas of the comb for size measurements. From the incubated brood combs, we removed daily the newly emerged individuals and freeze killed them followed by decapitation. The heads were subsequently photographed on top of graph paper and their width was measured with ImageJ software (ImageJ 1.46r). After each daily bee collection, we photographed the brood comb to track the progression of the bees emerging. It is noteworthy that *T. angustula* combs are not built symmetrically (Fig. 1b) and that the geometric centre of the comb does not necessarily correspond to the location where the first cells were constructed. In this study, we regard the cells that were first built (and from which the first bees emerge) as the centre. It took 7–10 days for all workers of a comb to emerge, depending on the size of the comb. We divided the comb into four different areas in relation to the time it took for all bees to emerge: the bees from the centre (area 1) emerged on days 1 and 2, while the bees from the outer ring (area 4) were the last to emerge, usually on days 6–10 (Fig. 1b, for sample sizes see Table 1). Areas 2 (outer centre) and 3 (periphery) were determined by halving the time period from day 3 to the day when bees started to emerge from the outer ring. This rough distinction into four different areas was sufficient to explore general patterns in worker size differences. Males were excluded from the data analysis.

Table 1 An overview of the mean head width of the guards caught at the entrances of the colonies (±s.d.), the mean head width of foragers, the mean head width of the newly emerged bees, the total number of measured newly emerged bees and percentages of guard-sized workers among the newly emerged bees for each colony

Colony	Head width of guards (mm)	Head width of foragers (mm)	Head width of newly emerged bees (mm)	Number of newly emerged bees measured				Guard-sized workers (%)
				Centre	Outer centre	Periphery	Outer ring	
A	1.89±0.04	1.79±0.02	1.77±0.04	78	177	147	36	4.11
B	1.94±0.03	1.81±0.04	1.83±0.04	104	56	67	14	5.81
C	1.81±0.04	1.71±0.04	1.74±0.04	94	113	84	29	5.63
D	1.81±0.05	1.74±0.02	1.72±0.03	99	133	129	72	3.70
E	1.85±0.03	1.75±0.04	1.72±0.04	134	111	271	34	1.45
Total				509	590	698	185	
Mean±s.d.								4.14±1.8

Estimating percentages of guard-sized bees for each colony

From each of the five colonies, we caught five standing and five hovering guards at the nest entrance (10 guards in total) and measured their head width as described above. With this data, we calculated the mean guard head width for each colony (Table 1). Subsequently, with the data we collected on the head sizes of newly emerged bees, we calculated for each colony and for each area on the brood comb the percentage of bees that emerge with a head width equal to or larger than the mean head width of the guards. This is a conservative measurement as it leaves out the guards that are smaller than the mean colony guard size. Therefore, we doubled the percentages because guard size is symmetrically distributed around its mean (Grüter et al. 2012; Fig. 2a). Although we cannot be certain that all the guard-sized workers will later perform guard duties, we deem it likely that most will do so, as guards in *T. angustula* are the largest workers (Grüter et al. 2012).

Weighing larval food from single cells

From five other colonies, we removed the youngest brood comb, which still contained unhatched eggs. To measure the weight of larval food in a brood cell, we uncapped it and removed the egg with forceps. Subsequently, we weighed the brood comb with the uncapped cell, which still contained all its larval food, to the nearest 0.1 mg using a Sartorius TE64 analytical balance. Afterwards, we removed the larval food with a capillary glass tube and absorbing paper and weighed the brood comb again. We measured the larval food content of 20 cells in the centre and 20 cells from the periphery of each comb.

Video analysis of cell provisioning

We prepared three medium-sized, queenright colonies in wooden, glass-covered observation hives inside the laboratory (different colonies than the ones used in all previous measurements). Colonies were connected to the outside via a plastic entrance tube. For the filming of POP, we removed the top parts of the wax sheets (involucrum) that surround the brood comb. We made sure that both cells at the centre and more at the outer areas of the brood comb were recorded. For colony 1, we obtained 98 observations of POP, 60 for colony 2 and 57 for colony 3 (215 in total). Videos were later analysed using the EthoLog 2.2 software (Ottoni 2000). Our main variable of interest was the number of nurse bees which regurgitate food in a cell. This behaviour is clearly visible as the bees contract their abdomen while leaning into a cell. The location of a cell was expressed as the number of rows from the location where the bees started to build a new comb (Fig. 1a), with row 1 being the cells at the very centre that were attached to a wax pillar the bees erected on top of the older comb. Because we were filming while the bees were progressively building the combs, we could easily count the number of rows a cell was away from the centre.

To determine if a brood cell receives more attention from nurse workers depending on its location (distance from the centre of the comb measured in cell rows), we counted the number of times nurses put their heads in the cell within 5 min before the cell was oviposited by the queen (cell inspections). As for some cells POP happened within the first 5 min on the video, we do not have this variable for all 215 cells (N=180). Additionally, we counted the cell inspections during POP, that is, the number of times nurse workers put their head in a cell without regurgitating after cell filling had begun. Nurse workers were not marked, and thus it was unknown if cell inspections were done by many or few different bees.

To examine if workers spend more time on POP for cells at different locations on the brood comb, we measured the time between the onset of cell filling and oviposition. This time of POP consists of (i) the duration of cell filling and (ii) the time between the end of cell filling and oviposition by the queen.

We noted when a worker laid a trophic egg to test whether the chance a trophic egg is laid by workers depends on cell location. Next to cell location, we included the time between

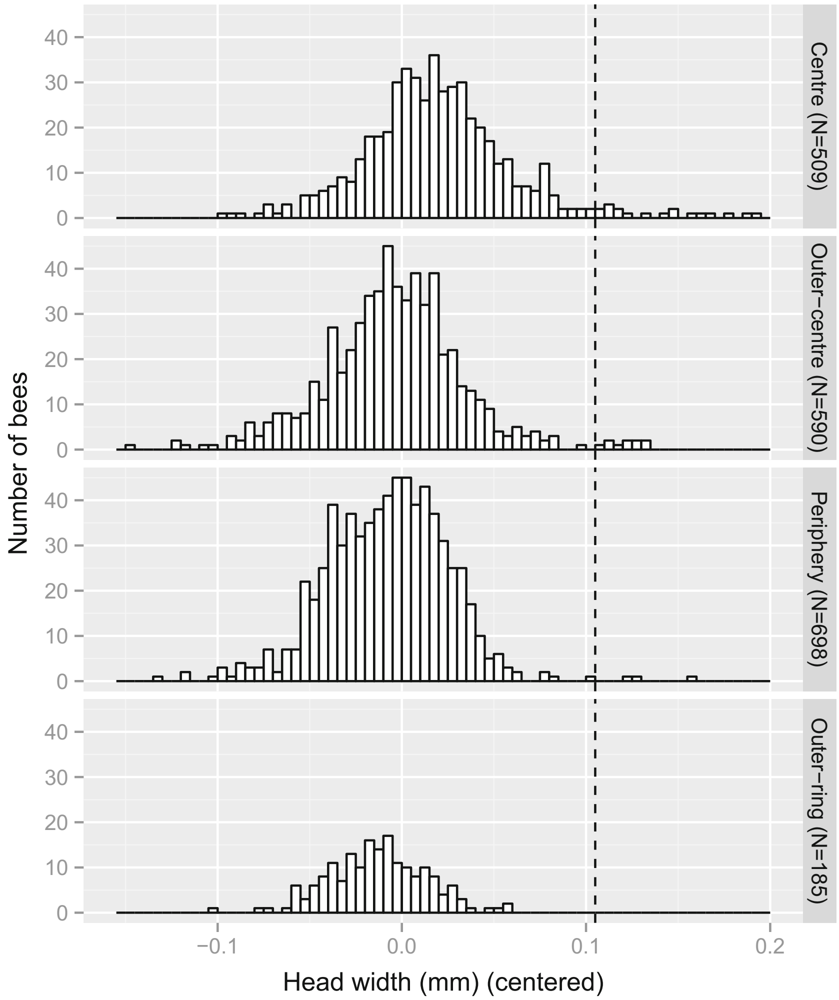

Fig. 2 The frequency distribution of emerging worker head widths for different areas of the brood comb (see also Fig. 1b). The values were centred by subtracting the colony mean. The *dashed line* indicates the mean head width of the guards caught at the entrances of the colonies (see also Table 1)

the end of cell filling and oviposition by the queen as explanatory variable in our statistical model (see below), as Koedam et al. (1997) found that the propensity of workers to lay a trophic egg increases with the duration of POP.

Males are produced year round in *T. angustula* with a peak in summer (Prato and Soares 2013). The proportion of males among newly emerged bees was low overall (2.0±1.2 s.d. %) and equal among the four brood comb areas, centre, outer centre, periphery, outer ring (*G* test, df=3, *G*=1.55, *p*= 0.67). Thus, it is unlikely that male eggs confound our data.

Data analysis

All data was analysed in R 2.9.2 (R Development Core Team 2009) using either generalized linear mixed effect models (GLMMs) or linear mixed effect models (LMEs), depending on the distribution of the response variable. Colony was always included as random effect to control for non-independence of data. The LMEs were fitted with restricted maximum likelihood (REML) using the lme function of the nlme package. For LME model selection, we used the step-down protocols suggested by Zuur et al. (2009): we first explored the best structure of the random components (comparing random intercept models with random intercept and slope models) using REML (tests not shown). To compare head width of workers between four different brood comb areas with LMEs, we used pair-wise comparisons and sequential Bonferroni correction to calculate *p* values (multcomp package in R). *T. angustula* colonies can differ in mean worker size (Grüter et al. 2012; see also Table 1, data based on 10 pollen foragers for each colony) which could obscure within colony differences in body size and brood cell measurements when data is averaged over colonies. Therefore, when we prepared the data for representation in figures, we centred the values of the data on the cell width, larval food content and emerging bee head width by subtracting the colony means.

To test for an effect of cell location on the number of larval food discharges by nurses into the cell, we ran a generalized linear mixed effect model (GLMM) with a Poisson error structure using the lmer function from the lme4 package. Colony was included as a random effect, and within colony, we nested the batch during which the egg was laid (see description of

POP above). A batch was defined as a series of cells being provisioned in short succession of each other. These batches start when the queen arrives on the brood comb and end when she leaves. We assumed that eggs which were filled in the same batch might have been more non-independent of each other because of a higher similarity in environmental conditions. In our dataset, batches ranged from 1 to 12 eggs. The independent variable location (number of rows/cells from the centre of the comb) was log-transformed because preliminary data analysis suggested that the relationship between location and larval food discharges was not linear.

To test whether the chance workers lay a trophic egg in a cell before oviposition by the queen depends on the location of the cell and the duration of POP, we used a GLMM with a binomial distribution. The interaction between the fixed effects was nonsignificant ($p>0.05$) and removed from the model. The main effects were kept in the model.

We used LMEs to test for an effect of location on (i) the time between the onset of cell filling and oviposition, (ii) the duration of cell filling and (iii) the time between the end of cell filling and oviposition. These nonindependent variables were square-root transformed to achieve normality.

Results

Head width of newly emerged bees

The frequency distributions of head widths show a wide range in head widths with the largest workers emerging from the centre of the comb (Fig. 2). Head width differed significantly among newly emerged bees from different areas on the brood comb (centre, 1.77±0.06 mm [mean±s.d.]; outer centre, 1.75±0.05 mm; periphery, 1.74±0.04 mm; outer ring 1.73±0.04 mm; LME, $N=1981$, $p<0.001$; Fig. 3a). Multiple comparison tests revealed that all areas of the comb differed significantly from each other, with bees emerging from the centre being significantly larger than bees from the other areas (Table 2; Fig. 3a).

The proportion of guard-sized workers was highest for the centre of the brood comb, while no guard-sized bees were found among the workers that emerged from the outer ring of the comb (Fig. 3b). The overall percentages of guard-sized workers that emerged ranged from 1.45 to 5.81 % for the five colonies (Table 1).

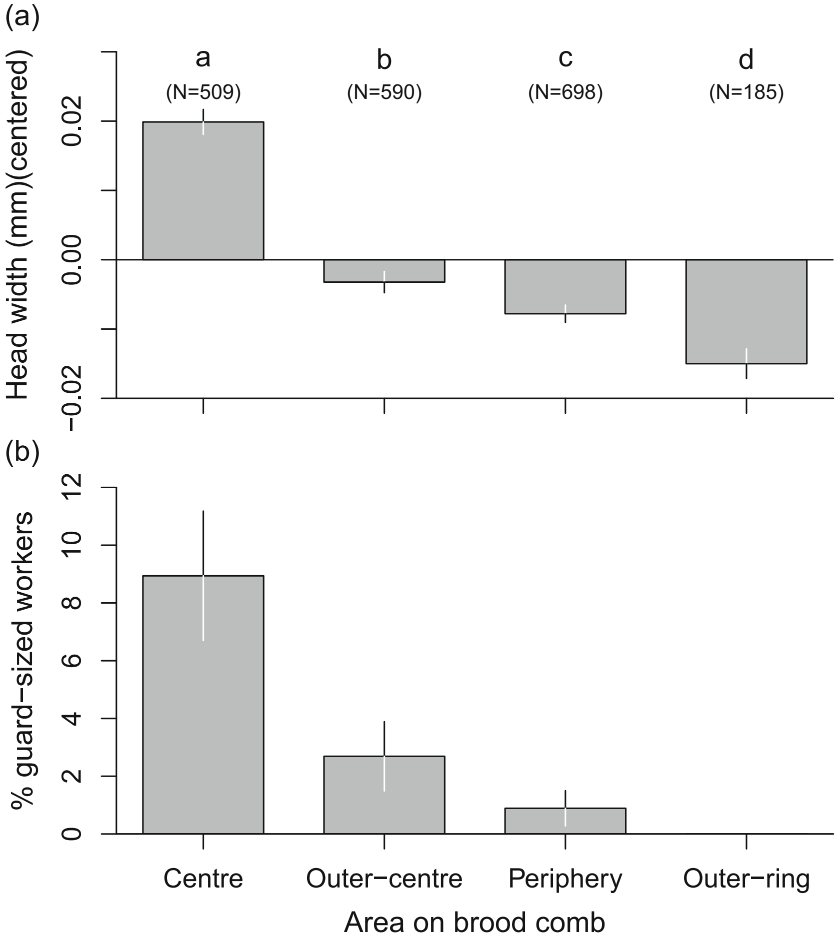

Fig. 3 The *bar plots* show for different areas on the comb: **a** the mean (±s.e.) head width (mm) of the newly emerged bees (centred by subtracting the colony mean) and **b** the mean (±s.e.) percentages of guard-sized bees estimated among the newly emerged bees. The *letters* above the *bars* in (**a**) indicate significant differences in mean head width between the bees from different comb areas. For sample sizes per area and per colony, see Table 1

Table 2 Results of the multiple comparisons of head widths of newly emerged bees between different areas on the brood comb (*p* values corrected with sequential Bonferroni)

Test	*z* value	*p*
Centre vs. outer centre	−10.77	<0.001
Centre vs. periphery	−13.33	<0.001
Centre vs. outer ring	−11.43	<0.001
Outer centre vs. periphery	−2.23	0.03
Outer centre vs. outer ring	−3.91	<0.001
Periphery vs. outer ring	2.44	0.03

Larval food and cell diameter

Cells from the centre of the comb had a significantly larger diameter (centre, 2.21±0.11 mm [mean±s.d.]; periphery, 2.15 ±0.07 mm; LME, $N=145$, $t=-4.51$, $p<0.001$). Although the effect size is small (<0.1 mm, Fig. 4a), cell width is a linear measure and the difference in cell volume between cells in the centre and the periphery of the comb is likely to be larger.

Additionally, cells in the centre of the comb contained on average 21.1 % more larval food than cells located in the periphery (10.9±1.9 [mean±s.d.] vs. 9.0±0.9 s.d. mg) (LME, $N=200$, $t=-11.80$, $p<0.001$, Fig. 4b).

Discharges

The results from the video analysis showed the same pattern: the location of a cell strongly affected the number of nurses that discharged larval food in the cell during POP (Fig. 5; GLMM, $N=215$, $z=-6.19$, $p<0.001$). Seemingly, only the cells at the very centre of the comb receive most larval food (Fig. 5). When we excluded the data from the cells that are in the first and second row of the comb, location no longer significantly affected the number of nurses that discharged in a cell (GLMM, $N=196$, $z=-0.90$, $p=0.37$).

The time between the onset of filling of a cell and the moment the queen lays an egg in the cell decreased with distance from the comb centre (LME, $N=212$, $t=-5.03$, $p<0.001$). This was caused by both the duration of cell filling

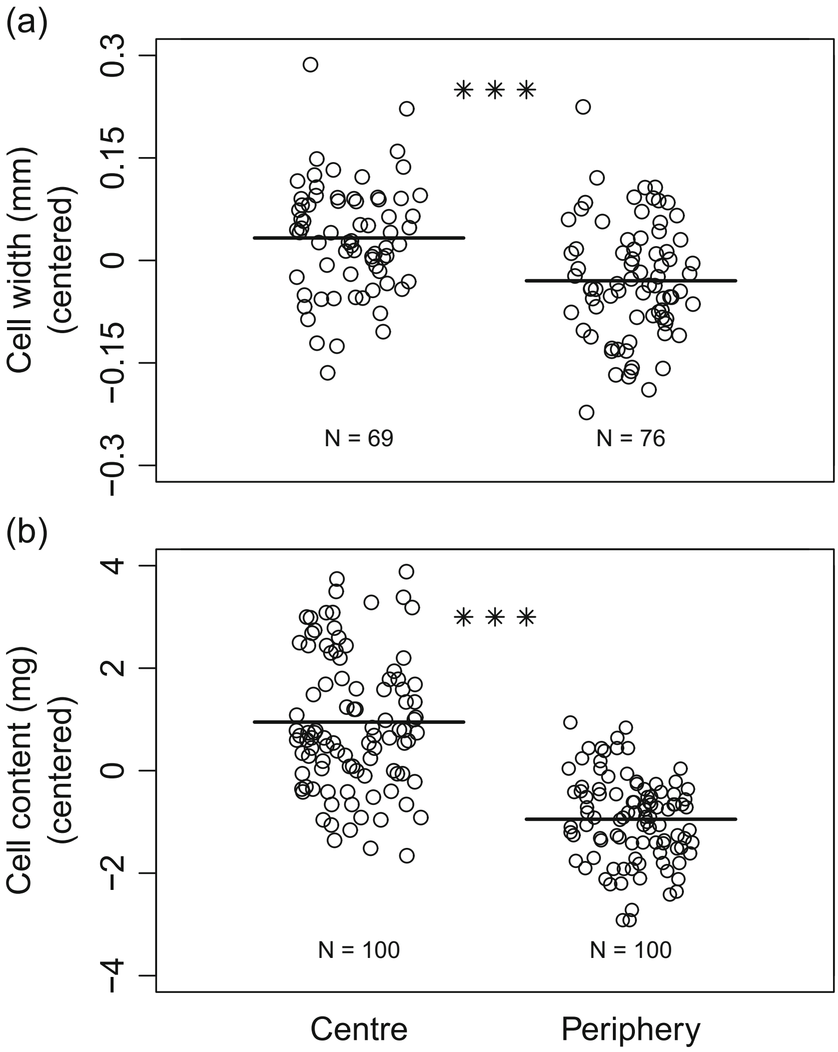

Fig. 4 **a** Cell width measurements (mm) and **b** the cell content measurements (mg). Both measurements were centred by subtracting the colony mean. The horizontal lines represent the means. Significant differences between the different comb areas are indicated by *asterisks*

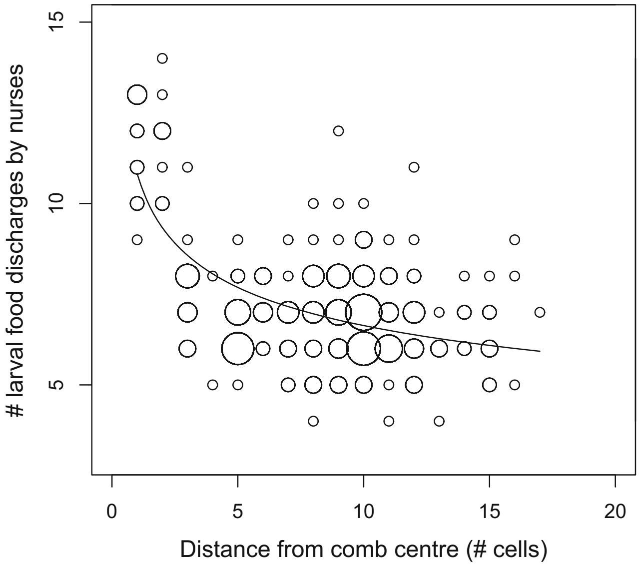

Fig. 5 The number of larval food discharged by nurses into the cells depending on their location. The curve is the best-fit line obtained by GLMM. The sizes of the circles represent the number of identical values. The *smallest circles* correspond with 1 value, and the *largest circle* indicates 14 identical values

(LME, N=215, t=−5.95, p<0.001), as well as the time between when the last nurse worker discharged into the cell and the moment of oviposition (LME, N=212, t=−2.41, p=0.02), being longer for more centrally located cells.

The longer it took for the queen to arrive to lay an egg after a cell was filled by the workers, the more likely it was that a worker would lay a trophic egg in the cell (GLMM, N=212, z=2.82, p=0.005). On the other hand, the location of the cell had no effect on the chance that a trophic egg was laid (z=0.28, p=0.78)

The number of cell inspections during POP decreased with distance from the comb centre (GLMM, N=213, z=−2.63, p=0.01). However, as the duration of POP is longer for more centrally located cells as shown above, the number of cell inspections per second was not affected by cell location (LME, N=212, t=−0.96, p=0.34). Similarly, the total number of inspections a cell received within 5 min before oviposition by the queen (before and during cell filling) was not affected by the cell's location (GLMM, N=180, z=−1.18, p=0.24).

Discussion

All our measurements strongly indicate that the largest workers in *T. angustula* predominantly emerge from the centre of the brood comb. Cells in the inner areas of the comb were wider and contained more larval food. Additionally, guard-sized workers emerged from these areas. Interestingly, our behavioural observations suggest that the cells in the very centre of the comb (row one to two) which are built first during comb construction are most important for soldier production. We propose that *T. angustula* workers build larger cells in the very centre of the comb and that therefore nursing workers need to deposit more larval food in these cells before they are filled to a level that is perceived as acceptable by nurse bees. It is yet unknown whether this is typical for all stingless bee species which build a similar horizontal comb or if it is unique to this species. Interestingly, in *Vespula* wasps and honey bees (*Apis mellifera*), nurses feed larvae in larger cells more and qualitatively different food which induces these larvae to develop into gynes (Spradbery 1973; Winston 1991). *T. angustula* also rears queens in large cells, which are built at the margin of the brood comb. These observations together with our results suggest an important role for cell size in the regulation of colony demography in these taxa. One of the questions that our finding raises is why some worker cells on the comb of *T. angustula* are larger than others. Is this done deliberately by the worker bees in order to raise soldiers or is this an inherent property of this comb structure? Possibly, larger cells are more frequent in the centre of the comb to give the comb more stability. A comparative approach could be used to determine if nest building and food distribution among larvae is done differently in stingless bee species without a soldier caste. A comparative study could also clarify whether other stingless bee species which build similar horizontal combs also build larger cells in the centre or if this is unique to *T. angustula*.

Our results suggest that food is an important factor in determining within colony size variation. In most stingless bee genera, the amount of food is the only factor responsible for queen determination (Hartfelder et al. 2006), and in general, food is important in regulating colony demography in bees (e.g. worker size variation, Couvillon and Dornhaus 2009; production of sexuals, Moo-Valle et al. 2001). But which factors determine differential feeding of larvae within a colony? Differences in the way of brood rearing have been linked to among species differences in worker size variation: *A. mellifera* workers are able to precisely regulate larval nutrition by progressively feeding small amounts and by inspecting frequently if a larva requires food (Huang and Otis 1991). This way of brood rearing is thought to cause the small among worker size variation typical for this species (Roulston and Cane 2000). In contrast, there is evidence from *Bombus* species that the method of food provisioning (mass provisioning in pocket making species vs. progressive provisioning in pollen storing species) is not a decisive factor in explaining the relatively large within colony size variation (e.g. Couvillon and Dornhaus 2009; Goulson 2010). Meliponini typically mass provision cells (Bego et al. 1999) and Couvillon and Dornhaus (2009) suggested that, unlike the large worker size variation they observed in a progressively feeding bumble bee, mass provisioning might be responsible for the small within colony worker size variation in Meliponini. Our results show that within the short time span of the mass provisioning of a cell by *T. angustula* nursing bees (average=25.4 s±1.0 SE, this study), variation in the amount of food which larvae receive can occur as some cells receive more than twice the amount of food compared to other cells (min. 4 and max. 14 discharges of larval food within the same colony). Thus, it seems that whether larvae are progressively or mass provisioned is not a decisive factor in generating among worker size variation in bees. On the other hand, there is strong evidence from the *Bombini* that worker/larva ratio influences the size of emerging bees (Couvillon and Dornhaus 2009; Shpigler et al. 2013). For example, in *B. impatiens*, larger workers develop in the nest centre, where the highest number of nursing bees tends to be found (Couvillon and Dornhaus 2009; Jandt and Dornhaus 2009). In *T. angustula*, however, nursing workers do not seem to be more present near cells located in the inner regions of the comb, as we found that cell attendance did not depend on location.

Food composition, mainly protein content, affected body size in the stingless bee *Nannotrigona perilampoides* (Quezada-Euán et al. 2011). However, this study measured differences in food composition throughout the year, and it is unknown whether variation in food composition generates within colony size variation in stingless bees. In honey bees, larval food is processed in the glands of workers, who progressively feed larvae small quantities of this secretion (Huang and Otis 1991). Most other bees provide an untreated pollen and nectar mixture to their offspring. Roulston and Cane (2000) suggested that the nutritional composition of glandular secretions may be more constant than a crude pollen and nectar mixture and that this could explain why worker honey bees are more uniform in size compared to other bee taxa. In *T. angustula*, a future study could examine if the composition of larval food (sugar and protein content) differs between cells depending on their location on the comb and if certain glandular products are added to the mix.

It is noteworthy that morphological differences in *T. angustula* workers are not only a size difference but also an allometric difference. This means that several body parts of *T. angustula* soldiers are proportionally different from foragers' (Grüter et al. 2012). The observation that soldiers differ allometrically from other workers suggests that the larger amount of food they receive in their brood cell initiates a developmental switch which results in a proportionally different growth rate of certain body parts of soldiers compared to other workers (Wheeler 1991; Shingleton et al. 2007). Such a developmental switch is likely to be a hormonal mediator which induces caste-specific changes in gene expression (Evans and Wheeler 2001; Tian and Zhou 2014). A technique for rearing of *T. angustula* workers in artificial cells has recently been developed (M. Prato, pers. comm.) and will allow rigorous study of the relationship between amount of larval food and growth of body parts in *T. angustula*.

It has yet to be clarified why worker size variation has led to the evolution of morphological subcastes in *T. angustula*, but not in other Meliponini and in *Bombus*, with the latter having a much larger size variation (e.g. up to eight-fold variance in body mass in *B. terrestris*, Goulson 2010, which is linked to variance in sensory abilities, e.g. Spaethe et al. 2007). One possible explanation could be the totipotency of *Bombus* workers, as in ants, the reproductive potential of individual workers has been suggested to be inversely related to colony complexity (Oster and Wilson 1978; Anderson and McShea 2001). Nursing workers of *T. angustula* possess working ovaries, but they have never been observed to lay viable eggs (Bego et al. 1999). Another explanation could be that the tasks which large and small bumble bee workers mainly perform (foraging and nursing, respectively) do not require special adaptations of body parts, while, for example, in *T. angustula* entrance guards might benefit from a relative increase in hind leg size while fighting with cleptobiotic bees (Grüter et al. 2012). Lastly, as bumble bees are equipped with a powerful sting, they might not need a specialised soldier caste for effective colony defence (Oster and Wilson 1978).

As there is an energetic cost to produce larger workers, caste ratio theory (Oster and Wilson 1978) predicts that investment in the soldier caste should depend on environmental conditions and colony needs (e.g. Passera et al. 1996). There are two important differences between how *T. angustula* and other eusocial insect groups raise their soldier caste, which might have major consequences for the ability of a *T. angustula* colony to adjust its caste ratios. First, in termites, aphids and ants, soldier production is regulated through positive and negative feedback mechanisms (e.g. Leveufe and Bordereau 1984; Shibao et al. 2004; Wheeler and Nijhout 1984). Often these feedback mechanisms work through pheromones which are transmitted to the larvae by direct contact with workers. In contrast, a stingless bee worker larva has no direct contact with adult workers because it develops in a sealed cell and therefore the up- or down-regulation of the soldier caste production does not depend on direct interactions between workers and larvae. Second, in ants, the latest larval instar can be induced to go into either a minor or major worker developmental trajectory (Wheeler and Nijhout 1981). Thus, an ant larva in a late developmental stage can respond to short-term changes in environmental cues and adjust its developmental trajectory in accordance to the needs of the colony. In contrast, in stingless bees the final size of a worker is predestined by the amount of food deposited by nurses in its cell before oviposition (Bego et al. 1999). If, for example, a *T. angustula* colony needs more soldiers due to an increased threat, it would take a colony at least 40 days (M. Prato, pers. comm.) to produce soldier-sized bees, which might take too long to protect the colony adequately. Thus, soldier rearing of

T. angustula results in a less flexible soldier caste ratio, but if it completely prevents an adaptive shift in worker caste ratio in response to changing environmental conditions has yet to be tested.

We estimated that about 4.14 % of the emerging *T. angustula* workers are guard-sized, with considerable variation among colonies (lowest 1.45 %, highest 5.81 %). Grüter et al. (2012) made an estimate of c. 2 %; however, we think that our new estimate is more accurate because we studied a larger number of workers, brood combs and colonies. This value is similar to what is known for some species of ants and termites with morphological soldier castes (e.g., 5–10 % in the harvester ant, *Pogonomyrmex badius* (Tschinkel 1998); 1–2 % in the termite *Cryptotermes domesticus* (Hasegawa 1997)). In the genus *Pheidole*, there is a large intra- and interspecific variation in soldier investment, ranging from 2 to 27 % (Yang et al. 2004; McGlynn et al. 2012). On the population, as well as the species level, variation in soldier investment was found to be related to the distribution of worker sizes in a colony in *Pheidole*: the proportion of soldiers was negatively related to the mean body size of the worker caste. That is, the threshold size at which worker larvae change their developmental trajectory and become soldiers is shifted downwards with a decrease in mean worker size (Wheeler and Nijhout 1981; Yang et al. 2004; McGlynn et al. 2012). Future studies can examine if this threshold shift model also applies to stingless bees.

In conclusion, soldier production in *T. angustula* is likely mediated through nutritional differences at the larval stage. Additionally, bee size seems to depend on the behaviour of nurse bees and (possibly even more) on cell-building bees. This suggests that larvae in *T. angustula* have much less control over their final worker size than ant larvae do. The existence of a soldier caste in bees allows us to make comparisons between different social insect taxa to increase our understanding of the proximate and ultimate mechanisms involved in the evolution of morphological castes. This study on the rearing of the soldier caste in *T. angustula* is a first step towards that aim.

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