

Repeated switches from cooperative to selfish worker oviposition during stingless bee evolution

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Abstract

Reproductive division of labour is a defining feature of insect societies. Stingless bees (Meliponini) are an interesting exception among the highly eusocial insects in that workers of many species contribute significantly to the production of males. Since workers remain sterile in other species of this large tropical tribe, it has been hypothesized that, in the latter species, ancestral queens have won the conflict over who produces the males. The fact that sterile workers of some species lay trophic eggs to feed the queen and display ritualized behaviours towards her during oviposition has been interpreted as an evolutionary relic of this ancient conflict. Here, I used ancestral state estimation to test whether worker reproduction is indeed the ancestral condition and worker sterility a derived state in stingless bees. Contrary to this hypothesis, data suggest that trophic egg laying was the ancestral condition, whereas selfish worker reproduction in queenright colonies evolved subsequently during stingless bee diversification. The appearance of worker reproduction in queenright conditions was tightly linked to the laying of trophic eggs, which suggests that having activated ovaries in queen presence facilitates the evolution of worker reproduction. Worker reproduction is also linked to brood cell architecture, but surprisingly not to colony size or queen–worker dimorphism. The reason for this association between brood cell architecture and worker oviposition is currently unknown. These results suggest that trophic eggs are not a relic of an ancient conflict, but a sign of overlapping interests between the queen and workers about who produces the males.

Introduction

Reproductive division of labour is one of the hallmarks of social insect biology (Wilson, 1971; Oster & Wilson, 1978; Hölldobler & Wilson, 1990). It means that a few individuals, and often just one, produce offspring, whereas most others are sterile workers that perform the labour that is required for colony maintenance, such as feeding the brood, defending the colony or foraging for resources. In the social hymenoptera, that is the ants and the social bees and wasps, workers are often able to develop their ovaries in the absence of a

queen and lay haploid eggs that develop into males (Wilson, 1971; Bourke, 1988; Ratnieks *et al.*, 2006). In the presence of the queen, however, queen pheromones inhibit the development of worker ovaries in many species (Endler *et al.*, 2004; Holman *et al.*, 2010; Nunes *et al.*, 2014; Van Oystaeyen *et al.*, 2014). If workers have developed ovaries in queenright conditions – as is the case, for example, in <0.1% of all workers in honeybees – and attempt to produce males, other workers or the queen often eat and kill these worker-laid eggs, so-called worker and queen policing (Ratnieks & Visscher, 1989; Ratnieks *et al.*, 2006; Wenseleers & Ratnieks, 2006; Zanette *et al.*, 2012). Worker policing is expected to evolve either because workers are more closely related to the queens' sons than to the sons of sisters (in polyandrous species) (Ratnieks, 1988; Ratnieks *et al.*, 2006) or because worker reproduction is costly for the colony, for example because reproductive

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workers neglect other duties, consume more energy or remove space for the rearing of worker brood (Ratnieks & Reeve, 1992; Tóth *et al.*, 2003; Hammond & Keller, 2004).

Stingless bees (Meliponini) are the largest (>500 species) and oldest (70–85 myo) group of eusocial bees (Rasmussen & Cameron, 2010). Colonies are usually headed by one singly mated queen and colony size varies from a few hundred to tens of thousands of individuals (Kerr *et al.*, 1962; Roubik, 1989; Tóth *et al.*, 2004). Stingless bees are unusual in that worker reproduction is common even in the presence of the queen. In some species, almost all males are produced by workers and in about half of the species workers contribute at least 10% of all males (Tóth *et al.*, 2004). Worker reproduction in stingless bees can be explained by a high relatedness among workers due to single mating (~0.75) (Paxton *et al.*, 1999; Peters *et al.*, 1999; Palmer *et al.*, 2002; Tóth *et al.*, 2002), which in turn reduces the incentives for workers to police each other (Ratnieks *et al.*, 2006). Another reason why worker reproduction could be more common in stingless bees is that cells are immediately sealed after mass-provisioning and oviposition, which prevent both the queen and workers from inspecting cells and removing worker-laid eggs (Sakagami, 1982).

However, there is a sizable number of stingless bee species where workers do not lay reproductive eggs in queenright conditions and in some species worker ovaries degenerate completely during development, making them incapable of reproduction (e.g. *Frieseomelitta* spp.) (Boleli *et al.*, 1999; Cruz-Landim, 2000). This could be explained by queen inhibition (e.g. through chemical manipulation or behavioural dominance) (Keller & Nonacs, 1993; Oi *et al.*, 2015; Grüter & Keller, 2016) or self-restraint by workers due to efficient worker policing (Keller & Nonacs, 1993; Wenseleers *et al.*, 2004a; Ratnieks & Wenseleers, 2005; Ratnieks *et al.*, 2006) or because the costs of worker reproduction to the colony outweigh the direct fitness benefits to the individual workers (Ratnieks & Reeve, 1992; Tóth *et al.*, 2004).

It has frequently been observed that stingless bee workers lay trophic eggs to feed the queen during the provisioning and oviposition process (POP) (Fig. 1; Movies S1 and S2) (Bassindale, 1955; Beig, 1972; Sakagami & Yamane, 1987; Wittmann *et al.*, 1991; Koedam *et al.*, 1996; Bego *et al.*, 1999; Segers *et al.*, 2015; Nunes *et al.*, 2017). The POP is also often accompanied by ritualized aggression between the queen and the workers (e.g. repeated darting towards the queen) (Sakagami *et al.*, 1973, 1977; Sakagami, 1982; Bego, 1990; Drummond *et al.*, 1996, 1999; Bego *et al.*, 1999). Both observations, trophic eggs and ritualized interactions, have been interpreted as evidence for an ancient conflict between queen and workers over male production (Hamilton, 1972; Crespi, 1992; Peters *et al.*, 1999; Oldroyd & Pratt, 2015), which was won by queens in the

ancestors of many present-day species. Accordingly, worker reproduction in queenright conditions is thought to represent the ancestral condition, whereas trophic egg production and worker sterility appeared subsequently. Sakagami *et al.* (1973), on the other hand, argued that undeveloped ovaries are the primitive state, whereas having activated ovaries to produce trophic and reproductive eggs is derived.

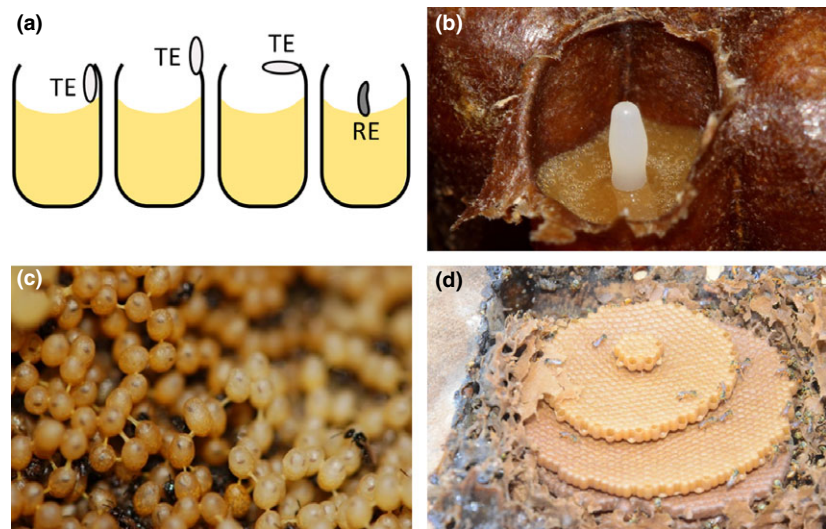
However, these hypotheses have not been formally explored due to long-standing uncertainties regarding the phylogenetic relationships among stingless bees. In recent years, several phylogenetic analyses have become available (Rasmussen & Cameron, 2007, 2010; Ramírez *et al.*, 2010), which make a formal investigation of the evolution of worker reproduction feasible. In a recent study, Nunes *et al.* (2017) found support for the hypothesis that activated ovaries and worker oviposition are the ancestral state in stingless bees (in contrast to the hypothesis proposed by Sakagami *et al.*, 1973), but since they did not differentiate between trophic egg laying to feed the queen and selfish worker reproduction, it remains unknown whether oviposition in stingless bee ancestors was selfish (reproductive eggs), as has been suggested, or cooperative (trophic eggs). Here, I used ancestral state estimation to explore whether worker reproduction or the laying of trophic eggs is ancestral or derived. The ‘conflict won by ancestral queens’ hypothesis predicts that worker reproduction in queenright conditions represents the ancestral state. Since worker reproduction has been suggested to correlate with both colony size and queen–worker size dimorphism (Bourke, 1999; Tóth *et al.*, 2004; Wenseleers *et al.*, 2004b; Fjerdingsstad & Crozier, 2006; Negróni *et al.*, 2016), I also tested the prediction that workers in species with large colonies and larger Q–W size dimorphism are less likely to reproduce (Bourke, 1999). This is expected because an increase in colony size should lower the reproductive potential of workers, for example due to increased selection on worker policing and requires an increase in morphological skew (Bourke, 1999; Wenseleers *et al.*, 2004b). Finally, I explored if the arrangement of brood cells is linked to worker reproduction. Brood cell architecture is likely to affect the ability of the queen and workers to manoeuvre and move from cell to cell, which in turn could potentially affect the ability of both queens and workers to prevent other workers from reproducing.

Materials and methods

Character states: worker reproduction

Different states of worker reproduction can be found in stingless bees (Table S1). In *Frieseomelitta* spp., for example, worker ovaries degenerate during development (Boleli *et al.*, 1999; Cruz-Landim, 2000) and workers are completely sterile. In other species, workers

Fig. 1 Oviposition and brood cell construction in stingless bees. (a) Depending on the species, workers can lay both trophic eggs (TE) and reproductive eggs (RE). Trophic eggs can be recognized by the position where they are laid (often on the rim or against the inside of the cell wall) and by their shape and size. (b) A normal queen-laid reproductive egg oviposited directly on the larval food in *Melipona fasciculata* (photograph by Cristiano Menezes). (c) A clustered arrangement of brood cells in *Leurotrigona muelleri* and (d) horizontal brood combs in *Tetragonisca fiebrigi* (photographs by C. Grüter).



do not lay eggs under queenright conditions, but start laying reproductive eggs under queenless conditions, for example as found in *Friesella schrottkyi* (Nunes *et al.*, 2014). A third situation is represented by species where workers lay both reproductive and trophic eggs under queenright conditions, as found in many *Melipona* species (Tóth *et al.*, 2004). Finally, there are species where workers only lay trophic eggs in queenright conditions (but workers often start to lay eggs in queenless conditions), for example as found in *Austroplebeia australis* (Drumond *et al.*, 1999). I found no case of a species where workers lay only reproductive eggs, but not trophic eggs in queenright conditions. Note that there are additional species with known worker reproduction in queenright conditions, for example in *Melipona marginata* (Tóth *et al.*, 2004), but information about the laying of trophic eggs was not available. These species were not included in the analysis. Trophic eggs can be distinguished from reproductive eggs by the position where they are laid (Movies S1 and S2) and by their size and shape (Fig. 1a,b).

I explored whether reproductive egg laying or trophic egg laying is likely to be the ancestral condition by performing two separate analyses. First, species were categorized based on whether workers lay reproductive eggs in queenright conditions (yes/no) and the ancestral state was estimated. Second, species were categorized based on whether workers lay trophic eggs in queenright conditions (yes/no) to estimate the ancestral condition.

Character states: brood cell architecture

There are two main types of brood cell architectures in stingless bees: clusters, which look like a bunch of grapes and, more frequently, horizontal disc-like combs (Fig. 1c,d). Less common is an intermediate

architecture called semi-combs (Kerr *et al.*, 1967; Brito *et al.*, 2012), a spiralled arrangement of cells and vertical combs (Schwarz, 1948; Oldroyd & Pratt, 2015). Since horizontal combs represent the most frequent arrangement, I classified species depending on whether they have horizontal combs (state 1) or another arrangement, mostly clusters (state 2). Horizontal combs are also likely to facilitate movement of bees compared to clusters and the more irregular semi-combs, which might affect the ability of workers to evade policing or prevent others from laying eggs.

Other continuous variables

Worker reproduction is likely to be linked to colony size and queen-worker dimorphism (Bourke, 1999; Wenseleers *et al.*, 2004b). Therefore, these data were collected from the available literature (Table S1).

Phylogenetic analysis

For phylogenetic relationships, I mainly used the phylogeny provided by Rasmussen & Cameron (2010). The chronogram from Rasmussen & Cameron (2010) was pruned to include only the species used in this study. For some relationships within the genera *Melipona* and *Plebeia*, more detailed analysis was used (Drumond *et al.*, 2000; Ramírez *et al.*, 2010; Werneck, 2016; Grüter *et al.*, 2017). Nonetheless, one assumption needed to be made based on available biological information. I assumed that *Tetragonula minangkabau* is phylogenetically close to *Tetragonula sarawakensis* (Sakagami & Inoue, 1985).

The resulting tree (Fig. 2) was used as a framework to estimate ancestral states of discrete characters. For the estimation of ancestral states, an MCMC approach was used for the stochastic reconstructions of character states (Huelsenbeck *et al.*, 2003; Revell, 2013). I ran

1000 simulations of a stochastic process of the character state changes across the tree branches, using empirical estimates for state frequencies for the prior distribution on the root node (assuming equal prior probabilities provided similar results). The posterior density of stochastically mapped character history was plotted on the phylogeny. To visualize the aggregate result of the 1000 stochastic maps, I used the function densityMap of the R-package phytools (Revell, 2012).

A second method for the estimation of ancestral character states for discretely valued traits using a continuous-time Markov chain model (Mk model) provided very similar results (shown in brackets in results and as pie charts in Fig. 2) to the MCMC approach. Mk models were performed with the ace function of the R-package ape (Paradis *et al.*, 2004).

I used Pagel's model to test for a correlated evolution of binary traits (Pagel, 1994) to test whether there is an association between (i) worker reproduction and the laying of trophic eggs; (ii) worker reproduction and brood cell architecture; and (iii) the laying of trophic eggs and brood cell architecture. To this end, I used the fitPagel function of the phytools package. Generalized least squares (GLS) models (Brownian motion model) that correct for phylogenetic dependence (Paradis, 2011) were used to test whether species with worker reproduction have larger colonies or a greater queen-worker size dimorphism.

Results

The ancestral state estimation suggests that trophic egg laying was the ancestral condition with a probability of $79.4 \pm 0.34\%$ ($\pm 95\%$ -confidence interval, $N = 1000$ simulations; rate parameter estimate = 0.0074) (81.3% using estimation based on Mk models; rate parameter estimate = 0.0076 ± 0.0057) vs. a $20.6 \pm 0.34\%$ (18.7% using estimation based on Mk models) probability that stingless bee ancestors did not lay trophic eggs (Fig. 2). Trophic egg laying was the most likely ancestral state in both New and Old World species but was lost five times in the species included in this study.

Then, I tested whether the laying of reproductive eggs in queenright conditions could have represented the ancestral state considering only the two states with or without reproductive egg laying. The analysis suggested only a $29.2 \pm 0.52\%$ (rate parameter estimate: 0.01) (23.1%; rate parameter estimate = 0.011 ± 0.008) probability that workers laid reproductive eggs ancestrally, with a $70.8 \pm 0.52\%$ (76.9%) probability that workers did not contribute to male production (Fig. 2). Based on the analysis, this was the case in both New and Old World species. However, worker reproduction in queenright colonies appeared six times in the species included in the study, most prominently in the largest stingless bee genus *Melipona*.

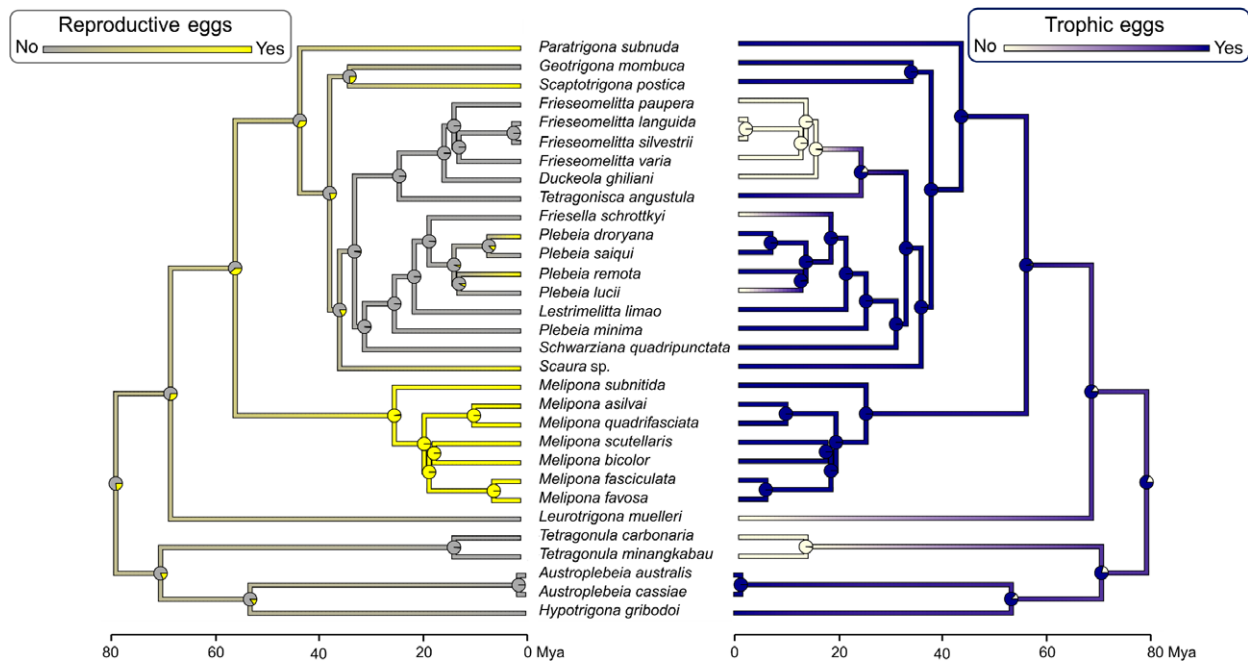


Fig. 2 Ancestral state estimation for the laying of reproductive eggs (yes/no) and trophic eggs (yes/no) in queenright conditions. The colour of branches is based on the MCMC approach. Pie charts provide estimates based on continuous-time Markov chain models. Overall, results were similar for both approaches. The chronogram is based on Rasmussen & Cameron (2010).

Species with worker reproduction are more likely to lay trophic eggs as well (Fig. 3a) (100% vs. 47.4% in species without worker reproduction). To test whether these two types of oviposition show signs of correlated evolution, Pagel's method was used. The test suggests that worker reproduction is indeed linked to the presence of trophic egg laying (likelihood ratio [LR] = 7.7, $P = 0.022$). Brood cell arrangement was also associated with worker reproduction (Table S1) as species with worker reproduction were more likely to build horizontal combs (Fig. 3b) (LR = 9.21, $P = 0.01$). Furthermore, there was a strong association between comb building and the laying of trophic eggs (Fig. 3c) (LR = 13.7, $P = 0.001$). Among species with trophic eggs, 78.9% (15 of 19) build horizontal combs, whereas none of the species without trophic eggs builds horizontal combs (zero of nine).

Then, I explored whether species with worker reproduction have larger colonies or a greater queen-worker size dimorphism, but found no significant relationships (worker reproduction and colony size: GLS, t -value = 0.81, $N = 26$, $P = 0.42$; worker reproduction and Q-W dimorphism: t -value = 0.45, $N = 17$, $P = 0.66$). However, there was a significant positive relationship between queen-worker dimorphism contrasts and colony size contrasts (t -value = 2.49, $R_{adj}^2 = 0.26$, $P = 0.026$).

Discussion

The ancestral state estimation suggests that the laying of trophic eggs to feed the queen during the provisioning and oviposition process (POP) represents the ancestral state with a 79.4–81.3% probability, whereas the laying of reproductive eggs most likely evolved later

(Fig. 3) (a 23.1–29.0% probability of representing the ancestral state). This challenges the hypothesis that worker reproduction in queenright colonies is the original condition in stingless bees and trophic eggs a leftover of an ancient queen-worker conflict (Sakagami *et al.*, 1973; Sakagami & Zucchi, 1974; Peters *et al.*, 1999). Thus, the ancestral condition seems to be best represented by species such as the Australian *Austroplebeia australis* or the American *Tetragonisca angustula*, where workers only lay trophic eggs under queenright conditions (Koedam *et al.*, 1996; Drumond *et al.*, 1999).

Worker reproduction in queenright colonies is also rare in honey bees and bumble bees (Ratnieks *et al.*, 2006; Zanette *et al.*, 2012), which suggests that workers of the common ancestor of all three eusocial corbiculate bee tribes (honeybees, bumblebees and stingless bees) contributed little or nothing to male production. The absence of worker reproduction could be the result of coercion (e.g. by the queen or workers) or because the rearing of the queen's sons was in the interest of workers. These results do not exclude the possibility that queens of an early corbiculate bee species won the conflict over male production, but they suggest that this would have happened before the appearance of stingless bees 70–85 mya (Rasmussen & Cameron, 2010; Cardinal & Danforth, 2011, 2013). In that case, what many researchers have described as ritualized queen-worker aggression during the POP could indeed represent a leftover of this conflict. However, the possibility that these queen-worker interactions do not represent ritualized aggression, but instead have communicative functions and help to coordinate the complex behavioural sequence during the POP remains an alternative explanation.

Worker reproduction under queenright conditions has appeared several times during stingless bee

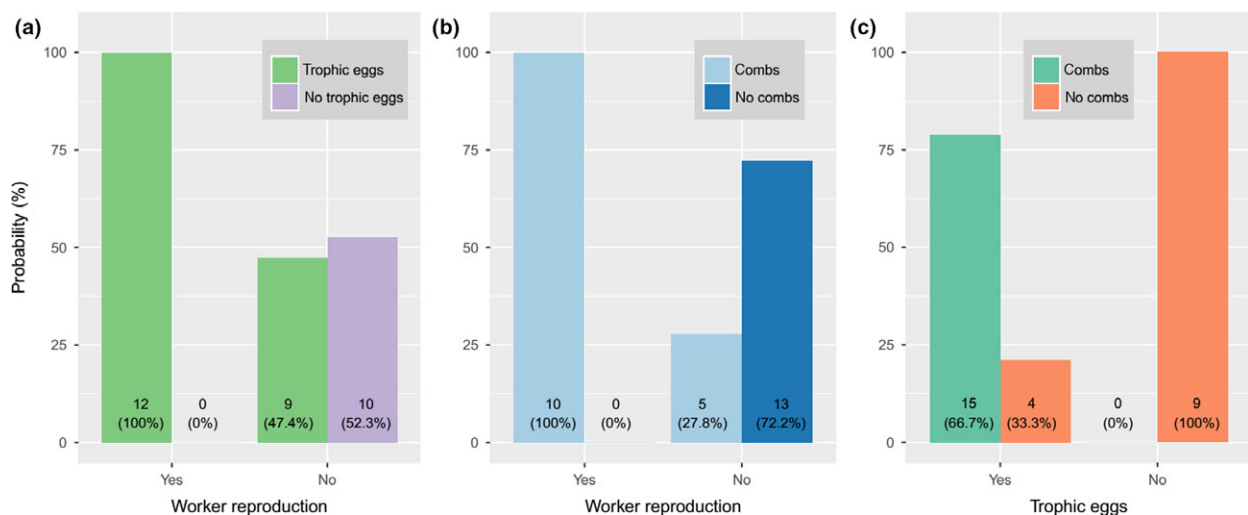


Fig. 3 Test of correlated evolution of binary traits. Numbers represent the number of species in each category. See Results Section for statistics.

evolution (Fig. 2). The laying of trophic eggs might have been an important precondition for worker reproduction to evolve: all species with worker reproduction also lay trophic eggs during the POP (Fig. 3). Laying trophic eggs implies that workers have activated ovaries under queenright conditions and that queens do not aggress oviposition workers. Both factors are likely to facilitate the switch from cooperative to selfish worker reproduction. Furthermore, only minor changes in the behavioural sequence of workers during the POP may have been necessary to lay reproductive eggs: instead of laying a trophic egg on the inside or on top of the collar of a finished brood cell just before queen oviposition (Fig. 1; Movie S1), workers lay an egg immediately after queen oviposition on the bottom of the brood cell (Beig, 1972; Sakagami, 1982). The worker laying a reproductive egg often also seals the cell, which makes it difficult for the queen and other workers to police cheating workers (Beig, 1972; Koedam, 1999; Koedam *et al.*, 2005; Velthuis *et al.*, 2005; Koedam & Imperatriz-Fonseca, 2012). As a result, cells can contain more than one larvae and Beig (1972) found that the male larvae (worker-laid) is usually larger, more mobile and eventually kills his female companion (queen-laid).

Why did selfish worker reproduction in the presence of an egg-laying queen evolve? It is unlikely that this is explained by changes in relatedness during evolutionary time as single mating and monogyny is ancestral to the group and the predominant situation found in present-day species (Strassmann, 2001; Tóth *et al.*, 2004; Hughes *et al.*, 2008). Worker sterility can be favoured even if worker reproduction seems beneficial to workers on relatedness grounds if worker reproduction leads to significant colony costs. This could be the case if reproductive workers neglect other duties, use up more resources, injure the queen during aggressive encounters or remove space for the rearing of workers (Ratnieks & Reeve, 1992; Tóth *et al.*, 2003). The differences among species in worker reproduction (Tóth *et al.*, 2004) indicate that the colony-level costs and individual-level benefits of worker reproduction differ greatly among species. The factors that could explain these species differences, however, are not well understood. For instance, it is not clear why *Geotrigona mombuca* only lays trophic eggs, whereas workers of its close relative *Scaptotrigona postica* commonly reproduce in the presence of the queen. Colony size has been suggested to both promote worker reproduction (Wenseleers *et al.*, 2004b), for example because colony-level costs might be lower in larger colonies or because queens are less able to prevent all workers from reproducing in larger colonies, and to select against worker reproduction, for example because worker policing is under stronger selection in larger colonies (Bourke, 1999; Tóth *et al.*, 2004; Fjerdingstad & Crozier, 2006). However, I found no relationship between colony size and worker reproduction. This

further supports the findings of Tóth *et al.* (2004) and Hammond & Keller (2004) and could be explained by opposing selection pressures: an increase in colony size might favour selfish egg laying due to reduced colony-level costs of worker reproduction (Wenseleers *et al.*, 2004b), but also require greater morphological specialization in both workers and queens and, therefore, greater morphological and reproductive skew (Bourke, 1999). The latter hypothesis is partly supported by the finding that larger queen–worker dimorphism predicts an increased colony size. The ‘queen power through size’ hypothesis predicts that relatively larger queens are more able to manipulate workers (Tóth *et al.*, 2004). However, I found no link between queen–worker dimorphism and worker reproduction. Stingless bee queens do not seem to have very strong mandibles and, thus, may have few means to forcibly prevent workers from reproducing (Tóth *et al.*, 2003). However, this analysis possibly lacks statistical power due to the fact that many species with worker reproduction are from the genus *Melipona*, which is also characterized by having an extremely small queen–worker size dimorphism.

Another factor that could explain differences in oviposition behaviour is the architecture of the brood combs. Species with horizontal combs were more likely to lay trophic and reproductive eggs in queenright conditions (Fig. 3). Interestingly, trophic eggs were lost in several species with clustered brood cell arrangements (*Frieseomelitta* and *Leurotrigona*). It is possible that the brood cell arrangement affects the POP in a way that either facilitates or complicates the laying of both trophic and reproductive eggs, but more research is needed to understand the links between brood cell architecture and worker reproduction.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Species used in this study. Information about brood cell architecture, colony size (data from Michener 1974; Roubik 1983; Tóth *et al.* 2004), queen-worker dimorphism (data from Tóth *et al.* 2004) and habitat is given

Movie S1 A worker deposits a trophic egg on the edge of an open cell (lower right of the image). Other workers form a circle around the cell with the trophic egg

Movie S2 A worker lays a trophic egg on the edge of an open cell (c. 4 s after start). The queen discovers the egg, eats it and oviposits an egg into the open brood cell

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