# Policing is more effective against eggs of non-natal versus natal workers at early colony stages in a bumblebee

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## Acknowledgements

We are grateful for practical assistance from members of AB's research group during experiments. We also thank the reviewer and editor for their comments improving the manuscript. This work was funded by the UK's Natural Environment Research Council [NERC research grant reference number NE/D003903/1], a NERC studentship held by JGH and a FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo – Brasil) studentship held by TN.

# Abstract

Eusocial insect colonies are vulnerable to exploitation by egg-laying workers arising either as natal reproductive workers or as non-natal reproductive 'drifting' workers (intraspecific social parasites). Worker egg-laying is potentially costly to the colony, but queens and workers can counter its costs via egg-eating (queen or worker policing). Bumblebee colonies exhibit egg laying by both natal and non-natal workers: natal workers collectively lay more eggs but do so only after a specific point in the colony cycle, the ‘competition point’, whereas non-natal workers potentially lay eggs throughout the colony cycle. These features create a special opportunity to investigate whether policing of worker-laid eggs is context-dependent (i.e. depends on worker origin of eggs and/or colony stage). We introduced artificial egg cells containing eggs laid by either natal or non-natal workers into colonies of the bumblebee *Bombus terrestris* both before and after their competition points, and observed the fate of introduced egg cells and eggs. In both colony stages, the majority of introduced egg cells and eggs were policed, demonstrating that policing was not activated only after the competition point. However, in the pre-competition point stage alone, a smaller proportion of non-natal workers' eggs (15%) remained after 20 h compared to the proportion of natal workers' eggs remaining (24%). More effective policing of non-natal workers' eggs early in the colony cycle potentially represents an adaptive, context-dependent response to the stage in the cycle when all worker-laid eggs are normally unrelated to the natal colony.

## Keywords

**Eusocial insects; Egg discrimination; Kin recognition; Policing; Social parasitism, Worker reproduction.**

## Significance

This study is novel because we investigate the importance of social context in determining the effectiveness of egg policing in social insect colonies. While previous studies have investigated the ability of social insects to ‘police’ worker-laid eggs from different sources, with policing occurring because it is in the policers' evolutionary interest, relatively little attention has been paid to whether or how the social context of policing affects its occurrence. In particular, by investigating the potentially separate effects of egg origin and colony stage, our study sheds light on both the mechanistic regulation of egg policing and its evolutionary benefits. Our results reveal that colony stage appears to be important for the policing of non-natal eggs and also suggests that social parasitism by non-natal workers influences the evolution of egg policing, at least in bumblebees.

## Statements and Declarations

The authors declare no competing interests. All experimental procedures followed the guidelines from the Association for the Study of Animal Behavior (ASAB) for the use of animals in research, and conformed to the legal requirements of the UK. The data supporting this study are openly available in Dryad (https://doi.org/10.5061/dryad.vhhmgqnzr).

## Introduction

Social groups, including those of eusocial insects, represent concentrations of resources that are vulnerable to exploitation by external and internal cheaters (Beekman and Oldroyd 2008; Bourke 2011; Ghoul et al. 2014; Lhomme and Hines 2018; Wenseleers et al. 2021). Cheating is frequently countered by the policing behaviours of other group members (Ratnieks 1988; Ratnieks and Wenseleers 2005; Singh and Boomsma 2015), but the effectiveness of policing behaviours in different contexts has been investigated in detail in relatively few systems (Endler et al. 2004; Karcher and Ratnieks 2014; Oi et al. 2015). In the eusocial Hymenoptera (ants, bees and wasps with a worker caste), a common form of cheating is egg-laying by reproductive workers. In many species, workers (all of which are female) possess ovaries and can produce viable male offspring from unfertilised, haploid eggs (Bourke 1988). Worker reproduction occurs in two contexts, both of which can be costly to the fitness of other colony members. The first context involves reproduction by natal workers (i.e. workers produced and eclosing in their own colony) and the second involves reproduction by non-natal workers (intraspecific social parasites) that originate from other colonies and enter host colonies via 'drifting' (Beekman and Oldroyd 2008). Reproduction by natal workers can be costly to nestmate fitness under the following conditions: (1) if other workers are more closely related to queen-produced males than to worker-produced males (Ratnieks 1988; Bourke and Franks 1995; Wenseleers and Ratnieks 2006); (2) if other workers favour female bias in the sex ratio (Foster and Ratnieks 2001b); and 3) if worker reproduction reduces colony productivity, e.g. by reducing foraging or nursing effort (Ratnieks 1988; Wenseleers et al. 2005a; Ohtsuki and Tsuji 2009; Teseo et al. 2013). Reproduction by non-natal workers frequently involves entry to host colonies followed by egg-laying by conspecific but unrelated workers, as documented among diverse eusocial Hymenoptera (Foitzik and Heinze 2000; Lopez-Vaamonde et al. 2004; Nanork et al. 2005; Hartel et al. 2006; Sumner et al. 2007; Chapman et al. 2010;

Zanette et al. 2014; Oliveira et al. 2016, 2021; Kuszewska et al. 2018; Nishimura and Ono 2021). Hence it is costly to host colony members because host workers then waste colony resources on rearing non-kin.

Both queens and workers in the eusocial Hymenoptera frequently police other individuals' reproduction, especially via egg eating, with queens typically eating workers' eggs (queen policing) and workers eating eggs laid by other workers (worker policing) (Ratnieks 1988; Wenseleers and Ratnieks 2006; Singh and Boomsma 2015). In many species, this policing acts as a key mechanism to remove eggs laid by natal workers (Van Doorn and Heringa 1986; Ratnieks and Visscher 1989; Monnin and Peeters 1997; Foster and Ratnieks 2001a; Wenseleers and Ratnieks 2006; Karcher and Ratnieks 2014; Wenseleers et al. 2021). The ability to discriminate natal worker-laid eggs from queen-laid eggs appears to depend on differences in chemical cues on the surfaces of eggs (Endler et al. 2004; van Zweden et al. 2009; Oi et al. 2015). Egg policing can also act as a key defence against social parasitism by non-natal workers (Gruter et al. 2018; Lhomme and Hines 2018), and both functions of egg policing could have been important for the evolution of egg eating in eusocial insect lineages (Lorenzi and Filippone 2000; Pirk et al. 2007; Beekman and Oldroyd 2008; Zanette et al. 2012). Consistent with policing of non-natal workers' eggs, there is evidence of an ability to discriminate non-natal worker-laid eggs in *Formica* ants (Helantera and Sundstrom 2007; Meunier et al. 2010; Chernenko et al. 2011) and the honey bee *Apis mellifera* (Pirk et al. 2007). However, some eggs of both natal and non-natal workers have been shown to escape policing in *A. mellifera* (Oldroyd and Ratnieks 2000; Martin et al. 2002), suggesting that egg discrimination is imperfect. Overall, the relative effectiveness of egg eating against the two forms of worker egg laying (by natal or non-natal workers) is not known and has not been studied in a single system.

The eusocial bumblebee *Bombus terrestris* exhibits both natal worker reproduction (e.g. Van Honk et al. 1981; Bloch 1999; Zanette et al. 2012) and reproduction by non-natal workers (e.g. Birmingham et al. 2004; Lopez-Vaamonde et al. 2004; Blacher et al. 2013a; O'Connor et al. 2013; Zanette et al. 2014) and so provides an excellent system in which to evaluate the relative effectiveness of egg eating in policing these two forms of worker reproduction. Both these forms are likely to co-occur more widely across the eusocial Hymenoptera, but currently their co-occurrence is known in only a few species, including *B. terrestris* and *A. mellifera* (Beekman and Oldroyd 2008). Bumblebee colonies exhibit an annual colony cycle in which a colony is produced by a single foundress queen and persists for a single season. Several weeks into the colony cycle, *B. terrestris* colonies exhibit a 'competition point', defined as the point at which some natal workers begin to lay eggs. This is likely, at a proximate level, to be triggered by changes in queen and/or nest chemical cues (Duchateau and Velthuis 1988; Bourke and Ratnieks 2001; Lopez-Vaamonde et al. 2007; Amsalem et al. 2009; Ayasse and Jarau 2014; Rottler-Hoermann et al. 2016; Almond et al. 2019). The competition point is characteristically accompanied by increased aggression between queen and workers and by the onset of queen and worker policing of natal worker-laid eggs, with the result that under 5% of natal worker-laid eggs survive for more than 2 hours after being laid (Zanette et al. 2012). There is evidence that chemical cues on both eggs and the wax egg cells in which they are laid allow workers to discriminate between natal worker-laid male eggs and queen-laid male eggs (Zanette et al. 2012). In *Bombus* spp. including *B. terrestris*, worker reproduction by socially parasitic non-natal workers has been documented in both semi-natural (Birmingham et al. 2004; Lopez-Vaamonde et al. 2004; Lefebvre and Pierre 2007) and field settings, although the frequency of non-natal workers in field colonies is low (1-3%) (Takahashi et al. 2010; O'Connor et al. 2013; Zanette et al. 2014). Lopez-Vaamonde *et al.* (2004) presented evidence that, by exhibiting elevated rates of aggression, egg-laying and reproductive success, *B. terrestris* non-natal workers exhibit a reproductive syndrome (suite of behaviours) differing from that shown by natal workers. This conclusion is supported by findings that fertile workers are more likely to remain fertile when introduced into a non-natal, host colony than when reintroduced into their natal colony (Yagound et al. 2012), and that workers drift to other nests more frequently when they are reproductively active and when their natal colony has passed the competition point (Blacher et al. 2013b). Combined, these findings suggest that, in *Bombus* species, worker drifting (either intentionally or as a result of navigation error) followed by intraspecific social parasitism represents a distinct, albeit low-frequency, evolutionary strategy via which workers may gain direct fitness.

The social biology of *B. terrestris* therefore provides a special opportunity to investigate whether the effectiveness of the colony (i.e. the summed efforts of the queen and workers) in detecting and policing worker-laid eggs is context-specific. Specifically, the effectiveness of egg policing could be influenced by the origin of workers' eggs (from natal or non-natal workers) and/or by stage in the colony cycle (colony stage). Colony stage is potentially influential because natal worker egg-laying in *B. terrestris* occurs only after the competition point (by definition), so before this point the only natal colony members' eggs present are queen-laid eggs, i.e. there are no (natal) workers' eggs to police. By contrast, drifting non-natal workers potentially enter colonies and lay eggs at any stage of the colony cycle. In particular, it is known that some eggs produced by non-natal workers are laid before their host colony's competition point and that these eggs may successfully develop into adult males (Lopez-Vaamonde et al. 2004). However, given the low frequency of non-natal workers observed in field colonies (see above), the chance of non-natal workers' eggs being present before their host colony's competition point is also likely to be low. For these reasons, queens and workers potentially activate egg policing only once the competition point has occurred. In this study, we therefore sought to determine the relative effectiveness of the colony's egg policing as a function of the origin of workers' eggs (natal or non-natal workers) and colony stage. We hypothesized that egg policing is more effective (1) against natal worker-laid eggs than against non-natal workers' eggs, i.e. because the former occur more frequently and so selection to police them is likely to have been stronger, and (2) after the competition point than before the competition point, i.e. because natal workers lay eggs only after the competition point, and non-natal workers' eggs are likely to be rare, suggesting policing yields greatest benefits after the competition point. Accordingly, we employed a factorial design, introducing eggs of either natal or non-natal worker origin to *B. terrestris* colonies both before and after the receiving colonies' competition point.

## Materials and Methods

*Colony maintenance and microcolony construction*

Ten pre-competition point *B. terrestris terrestris* colonies were obtained from a commercial company (Syngenta Bioline, Weert, The Netherlands). The colonies were queenright (containing the foundress queen) upon receipt. Colonies were maintained in plastic nest boxes provided by the supplier (20 cm × 20 cm × 15 cm), in standard conditions (constant darkness, approximately 28°C, 50% relative humidity) for the duration of the experiment, with all observations and manipulations being conducted under dim red light. These colonies and all experimental bees were fed *ad libitum* with pollen and sugar syrup (‘Attracker’; Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) throughout the experiment. The 10 colonies were divided randomly into 5 pairs and labelled accordingly. To obtain worker-laid eggs, workers and cocoons (brood cells each containing a pupa or late-stage larva) were removed from each colony and placed into small (140 × 79 × 60 mm) plastic boxes (hereafter, 'microcolonies'). Three microcolonies were constructed per colony of origin, with each containing 3-5 workers and 5 cocoons. Such microcolony conditions induce workers to lay eggs (e.g. Alaux et al. 2007; Amsalem and Hefetz 2011). To maintain at least 3 workers per microcolony, workers that died were replaced by additional workers randomly selected from the colony of origin. Once workers in at least one microcolony per colony of origin had begun to lay eggs (9 days after microcolonies had been established), egg cell introductions were commenced, with this day classed as the beginning of the experiment for all colonies. The total duration of the experiment, from first to last introduction, was 17 days.

### *Egg cell introduction: experimental set-up*

Egg cell introductions of eggs derived from the microcolonies into the ten whole colonies were conducted to test the response of pre- and post-competition point colonies to eggs laid by either natal or non-natal workers. For each egg cell introduction, one investigator (JGH) introduced the contents of one or more egg cells taken from a microcolony to a manually-constructed artificial egg cell, with a single artificial cell containing several eggs then being used for each introduction. The total number of egg cell introductions was 119, consisting of 744 eggs overall (mean ± SD = 6.3 ± 1.5 eggs per introduced egg cell). In order to standardise across treatments, artificial egg cells were constructed from wax taken from an empty cell in the receiving colony on the day of the introduction and were designed to resemble *B. terrestris* egg cells in size and shape. The workers in the microcolony from which the eggs were taken were originally from either the receiving colony (natal egg origin) or another colony (non-natal egg origin). Where possible, non-natal eggs introduced to a receiving colony were sourced from a microcolony whose workers originated from the colony with which the receiving colony had been paired (109 of 119 introductions). This pairing protocol was used to keep the number of introduced eggs sourced from each colony approximately equal, so reducing any effect of colony of origin. On dates when microcolonies from a paired colony failed to provide sufficient eggs for introduction to a receiving colony, eggs were instead taken from a microcolony derived from another colony. Where this was not possible (due to insufficient eggs), no introduction into the receiving colony was performed on that day. In placing eggs in the artificial egg cell, care was taken to ensure that wax from the worker-made egg cell in the microcolony did not contaminate the wax used to construct the artificial egg cell, and that both the cell and eggs only came into contact with the tools used (cocktail sticks and forceps) and nitrile gloves, which were replaced between each introduction. Care was also taken to avoid crushing the eggs when sealing the cell. After each artificial egg cell (containing the eggs) had been sealed, the cell was then placed and fixed on top of the brood cells in the receiving colony in a visible position. As far as was possible, introductions (111 of 119 introductions) were carried out over sets of two consecutive days, with a colony receiving egg cells in the natal treatment on the first day, and in the non-natal treatment on the second day (or vice versa, in a randomly determined order). This helped to ensure that treatments were equally represented across time for each colony. The remaining 8 of 119 introductions were performed at other times throughout the experiment (fig. 2). All ten colonies received six natal introductions and six non-natal introductions with the exception of one colony that received only five natal (and six non-natal) introductions. No more than one introduction per day was conducted in any one colony, and all introductions were performed between 14:00 and 18:00.

Since we wished to test whether the response of colonies to eggs differed according to colony stage (i.e. whether before or after competition point), each of the ten whole colonies was monitored regularly (every 1-2 days) to check whether it had reached the competition point. Following Duchateau et al. (2004), a colony was considered to have reached the competition point when at least one of four criteria was observed (multiple open egg cells, egg-eating by queen or workers, aggression between queen and workers, or egg-laying by workers), except that the opening of introduced experimental egg cells or the eating of eggs from them were excluded (since such behaviours may have been the result of the experimental conditions and did not necessarily demonstrate egg-laying by workers in the receiving colony). In two colonies, the competition point occurred at, or before, the start of the experiment. In another one colony, the competition point had not occurred by the end of the experiment. In the remaining seven colonies, the competition point (CP) occurred during the experiment (mean ± SD = 8 ± 4 days into the 17-day experimental period). The egg cell introductions were performed both before and after the competition point, creating four experimental conditions as follows: natal worker eggs introduced into pre-competition point colonies (n = 19 egg cells introduced), non-natal worker eggs introduced into pre-competition point colonies (n = 22 egg cells introduced), natal worker eggs introduced into post-competition point colonies (n = 40 egg cells introduced), and non-natal worker eggs introduced into post-competition point colonies (n = 38 egg cells introduced). We also monitored the colonies for the death of the queen, which occurred during the experiment in three colonies (2a, day 12; 2b, day 2; 4a, day 1).

### *Egg cell introduction: assessment of egg cell fate*

The fate of introduced egg cells (including their contained eggs) was assessed in three ways. First, to investigate the initial time course of policing, and to measure egg cell fate in the period shortly after introduction, we determined, at intervals, the rate of egg cell ‘destruction’ over the first 3 hours since introduction, with a 'destroyed' egg cell being defined as one that had been opened and then left open with no eggs remaining. For this, after each egg cell introduction, the introduced egg cell was observed at 30-minute intervals over 3 hours to check it for destruction. Second, to investigate the longer term fate of egg cells, we determined whether the cells had been destroyed or not after 20 hours since introduction. Third, to account for the fate of egg cells that, after 20 hours, had survived destruction, we determined the proportion of eggs remaining in each of these cells. For this, we removed surviving cells from the colony and counted the number of eggs remaining inside. The proportion of eggs remaining was calculated, over all introduced egg cells (i.e. both those destroyed after 20 hours, which returned values of zero, and those surviving destruction after 20 hours), as (number of eggs remaining) / (number of eggs introduced). Occasionally, it was observed that eggs had been laid in the introduced egg cell, and this was also shown by data from digital filming (see below). Although it was not possible for the investigator to discriminate between such eggs and eggs present when the egg cell was introduced, egg-laying in the introduced egg cells was unlikely to have affected the conclusions because a) it was relatively rare (occurring in 2 of 15 digitally filmed introductions; see *Results*), and b) a relatively large proportion of egg cells were destroyed (see *Results*), so these would have been scored as destroyed even if they had contained eggs laid since the introduction of the egg cell. All assessments of egg cell fate, including counts of eggs remaining, were conducted blindly by a second investigator (TN), who was naïve as to the treatment of each cell.

### *Egg cell introduction: digital filming of adult behaviour at introduced egg cells*

To check that eggs that went missing from introduced egg cells were in fact eaten, a sample of introduced egg cells were also digitally filmed for 10 hours (or until the cell was destroyed) following introduction. Specifically, fifteen introduced egg cells (3 natal, pre-competition point, 2 non-natal, pre-competition point, 4 natal, post-competition point and 6 non-natal, post-competition point) were filmed using digital camcorders (Sony DCR-SR32). Filming began within 30 minutes of the egg cell introduction, after setting up the camcorders to focus on introduced cells. Seven introductions were filmed at the start of the experiment (day 1 or 2), and eight introductions were filmed at the end of experiment (day 15 or 16). Each colony was filmed 1-2 times. The digital films were then viewed to score all instances in which introduced egg cells were opened, egg eating from them visibly occurred or egg laying into them visibly occurred. This allowed us both to check whether egg eating was the cause of the reduction in egg numbers in introduced egg cells and to estimate the extent of egg laying (if any) into introduced egg cells. The caste (queen or worker) of individuals performing these actions was also scored. Finally, because egg eating may have sometimes occurred without being visible in the digital film (due to the cell's mouth being obscured), and to refine our estimate of the relative proportions of queen and worker policing, a subset of filmed introductions (three at the start and three at the end) were analysed in more detail to measure the time over which either queens or workers spent 'engaged' at the egg cell, i.e. with the head inserted into the cell (indicating either egg eating or another behaviour such as inspection of the egg cell's interior).

### *Statistical Analyses*

The effect of egg origin (whether introduced eggs were laid by a natal or non-natal worker) and colony stage (whether receiving colony was pre- or post-competition point) on the rate at which introduced egg cells were destroyed over the first 3 hours following introduction was tested using a mixed effects Cox (Proportional Hazards) model fitted by maximum likelihood. Egg origin, colony stage and their interaction were used as explanatory variables. Colony identity was used as a random intercept factor to account for the repeated use of each colony for the experimental egg cell introductions. The event time was the time elapsed between introduction and the earliest time (in intervals of 30 min) at which the introduced egg cell was observed to have been destroyed (i.e. opened and containing no eggs). Any cells opened but still containing eggs were classified as not destroyed. Cells not destroyed within 3 hours were included as right-censored data (i.e. as still informative because the cells were not destroyed before this time).

Generalised linear mixed models (GLMMs), with a zero-inflated binomial error distribution and logit link function, were used to evaluate the effects of egg origin and colony stage on egg cell fate at 20 hours. Whether or not the egg cell was destroyed within 20 hours of introduction was used as a binary response variable, and egg origin, colony stage and their interaction were used as fixed factors. Colony identity was used as a random (intercept) factor to account for the multiple measurements of egg policing obtained from each receiving colony.

Finally, another set of GLMMs was used to test the effect of egg origin and colony stage on the proportion of eggs remaining within egg cells after 20 hours. These were conducted with the same specifications as the previous GLMMs, but with the proportion of eggs remaining as the response variable.

Model selection was based on delta AICc and model weight values for all possible combinations of explanatory variables in each set of GLMMs, including null models with the intercept only (Burnham et al. 2011; Grueber et al. 2011). Models with a delta AICc below 4 were considered to be equally supported candidate models (Burnham et al. 2011). Model-averaged parameter estimates (and error) for all fixed terms in the candidate set were obtained using a natural average method (Grueber et al. 2011), and variables with a coefficient with a CI (95%) not overlapping zero were considered to have a stronger effect.

All statistical analyses were performed using R versions 2.15.2 or above (R Core Team 2012, 2022), using the survival (Therneau 2015), coxme (Therneau, 2022), lme4 (Bates et al. 2015), glmmTMB (Fournier et al. 2012; Skaug et al. 2013; Brooks et al. 2017), MuMIn (Barton 2019) and DHARMa (Hartig 2020) packages.

# Results

## *Fate of introduced egg cells*

The analysis of the rate of egg cell destruction within the first 3 hours after introduction detected no effects of egg origin (laid by natal or non-natal worker) or colony stage (receiving colony is pre- or post-competition point). Specifically, this rate was not significantly affected by egg origin (Cox model coefficient = 0.038 , $z =$ 0.11, , $p = 0.91$), colony stage (coefficient = -0.232, $z = -0.52$, $p = 0.6$), or the interaction of these terms (coefficient = -0.321, $z = -052$, $p = 0.6$; fig 1). However, there was an effect of colony (random intercept s.d. = 0.543, deviance difference = 2.45, $\chi^2 = 4.892$, d.f.= 1, $p = 0.026$), suggesting the existence of variation across colonies in the effectiveness of their policing.

The analysis of the rate of egg cell destruction at 20 hours after introduction also detected no effects of egg origin or colony stage. Specifically, when modelling egg cell destruction at 20 hours as a response variable, GLMMs with a fixed interaction term of egg origin × colony stage were not retained in the candidate set of models. Models with egg origin and/or colony stage as fixed terms received the same support as the intercept-only model (delta AICc < 4). This latter result indicated that egg origin and colony stage did not have a strong effect on egg cell fate at 20 hours (model averaged parameter ± CI: 0.22 ± 0.892 and 0.130 ± 1.61, respectively; fig. 2).

By contrast, the analysis of the proportion of eggs remaining in egg cells after 20 hours found that this proportion was lower for eggs laid by non-natal workers, but only if introduced to pre-competition point colonies. GLMMs including egg origin, colony stage and their interaction as fixed terms were retained in the candidate set of models (delta AICc < 4). The intercept-only model, and the model with colony stage as the single fixed term, were not retained. Estimates of averaged fixed term coefficients indicated that the interaction of egg origin × colony stage had a strong effect (model averaged parameter ± CI: egg origin -0.321 ± 0.321, colony stage 0.261 ± 0.513, interaction -0.671 ± 0635). Specifically, in pre-competition point colonies, but not post-competition point ones, the proportion of eggs remaining in introduced egg cells after 20 h was lower for non-natal eggs (0.15) than for natal eggs (0.24) (fig.3). In post-competition point colonies, the corresponding proportions were 0.27 and 0.28 for non-natal and natal eggs, respectively (fig. 3).

In two colonies (2b and 4a), the queens died early in the experiment and no egg cell introductions were performed before the queen died, meaning queenlessness may have affected the results. For all models, removing these two colonies did not change the candidate set of models or the magnitude of the effect of the egg origin × colony stage interaction, suggesting that early queen death had no discernible impact on the findings.

### *Adult behaviour at introduced egg cells*

Among the 15 introductions in which adult behaviour at introduced egg cells was digitally filmed, two involved egg cells that had no eggs present at the start of filming (presumably because egg-eating had already occurred in the <30 minutes between introduction and the start of filming). Of the remaining 13 introductions, one ended with all eggs remaining after 20 hours. Among the other 12 introductions (i.e. with eggs present at the start of filming and fewer left at the end), egg eating from the introduced egg cells was observed in all cases. Specifically, egg eating by both workers and the queen was observed in 2 cases, egg eating by the workers alone was observed in a further 8 cases, and egg eating by the queen alone was observed in a further 2 cases. Hence, workers accounted for an estimated (2 × 0.5) + (8 × 1.0) + (2 × 0.0) = 9 of 12 observed egg-eating events (75%). Observed egg eating was always preceded by the queen or workers opening the cell. For the six filmed introductions analysed for engagement time, the total amounts of queen and worker engagement were 601 s and 1901 s, respectively. Therefore, consistent with the observation data, workers accounted for 76% of engagement behaviour at egg cells. We conclude that, in the experiment as a whole, workers performed approximately 75% of all egg eating and queens performed approximately 25% of all egg eating.

Worker egg-laying into the introduced egg cell was observed in one of the 15 introductions in which adult behaviour at the introduced egg cell was filmed, and queen egg-laying into the introduced egg cell was observed in a second such introduction. Because egg-laying in these cases was partially obscured by other bees, and multiple eggs may have been laid in any one session, it was not possible to determine the number of eggs laid during filming. In these two cases (which represent 13% of introductions followed by filming), no eggs remained in the cells at 20 hours. However, if egg-laying occurred in other cases, it is possible that these eggs could have remained in introduced egg cells until 20 hours, and artificially inflated the number of eggs remaining. Since some non-natal eggs would have been replaced with natal ones, such an occurrence might have affected the conclusions regarding the effect of egg origin. We therefore re-ran the relevant models on our data but with a simulated removal of post-introduction egg-laying up to the observed level, by subtracting a variable proportion of the eggs remaining (0 – 13%) following each non-natal introduction, and found that our results were not affected. Hence egg-laying into introduced egg cells at the observed level did not affect our conclusions.

In all 15 introductions followed by filming, workers and/or queens were observed opening the cell to expose eggs, including four cases in which some eggs remained after 20 hours, suggesting that the artificial cells did not prevent individuals from accessing and eating eggs throughout the experimental introductions.

## Discussion

To test the effectiveness of policing of worker-laid eggs as a function of egg origin and colony stage, we introduced egg cells containing eggs laid by natal and non-natal workers into pre- and post-competition point *B. terrestris* colonies. We found that the complete destruction of introduced egg cells (i.e. such that cells had no surviving eggs) did not depend on egg origin or colony stage, as there were no differences in the initial rate (i.e. rate over the first 3 hours) of egg cell destruction or the frequency of egg cells destroyed after 20 hours. However, we found that the mean proportion of surviving eggs differed with egg origin for introductions into pre-competition point colonies. Specifically, the mean proportion of introduced eggs per cell surviving after 20 hours was 15% for non-natal eggs, compared to 24% for natal eggs. No such difference was found in introductions into post-competition point colonies. Therefore, policing by colonies (principally workers) of non-natal worker-laid eggs was more effective than their policing of natal worker-laid eggs, but only during the pre-competition point stage of the colony cycle. Moreover, the results show that policing of eggs in *B. terrestris* is not a behaviour that is activated only once the colony has passed its competition point.

Intriguingly, these results provide evidence of an interaction between egg origin and colony stage on the effectiveness of egg policing, but not in the direction predicted by our initial hypotheses. One reason why workers might police eggs from non-natal workers more effectively in pre-competition point colonies is that disruption to colony efficiency could be particularly costly while the colony is still growing and before it has produced sexuals (new queens and males). Colonies do not apparently have greater overall sensitivity towards the presence of worker-laid eggs during this time, since the survival of natal worker-laid eggs was similar before (24%) and after the competition point (28%). However, as natal worker-laid eggs are not laid before the competition point in nature, there may have been selection for increased sensitivity towards non-natal eggs during this colony stage only, since any worker-laid eggs present would normally be unrelated ones laid by socially parasitic drifter workers from other colonies. Additionally, it is possible that the threat of cuckoo bumblebees, congeneric interspecific social parasites that parasitise bumblebee colonies by usurping the queen and laying eggs (Goulson 2010), may also have increased the sensitivity towards eggs laid by non-natal females during the colony's growth period. In other eusocial Hymenoptera, worker discrimination of non-natal eggs has also been reported, but without the social context of colony stage being investigated (Lorenzi and Filippone 2000; Helanterä and Sundström 2007; Pirk et al. 2007; Meunier et al. 2010; Chernenko et al. 2011).

Our study demonstrates that, in the pre-competition point stage, *B. terrestris* colonies can detect non-natal worker-laid eggs and police them more effectively, which represents a form of both egg discrimination and kin recognition. The mechanism used to achieve this remains unknown, but, in line with previous studies (see above), almost certainly involves detecting differences in chemical cues on the egg surface. It is also worth noting that, unlike the case in other examples of worker-laid eggs escaping policing (Oldroyd and Ratnieks 2000; Martin et al. 2002), the relatively small magnitude of the discrimination against non-natal worker-laid eggs in the current study is unlikely to have arisen through workers chemically disguising eggs, as has been hypothesised (e.g. Amsalem et al. 2015; Gruter et al. 2018). This is because non-natal (as well as natal) eggs were taken from isolated groups of workers and not from workers that had chosen to enter non-natal colonies. In addition to the policing of worker-laid eggs, other behaviours may also help workers prevent the production of worker-produced males (Gruter et al. 2018; Lhomme and Hines 2018), such as aggression towards reproductive natal or non-natal workers (Duchateau and Velthuis 1988; Lopez-Vaamonde et al. 2004; Blacher et al. 2013a). Evidently, even in combination, policing behaviours are not always completely successful, as demonstrated, in *B. terrestris*, by some worker-laid eggs escaping policing (Zanette et al. 2012; current study) and by the existence of worker-produced adult males of both natal and non-natal origin in field or semi-natural colonies (Lopez-Vaamonde et al. 2004; Takahashi et al. 2010; O'Connor et al. 2013).

There are several ways in which the experimental design and the commercial colonies used in our study might have affected the results found. First, receiving colonies might have found it harder to discriminate natal and non-natal workers' eggs because being kept in microcolonies had caused natal workers to lay eggs lacking chemical cues signalling their natal origin. However, discrimination did occur in pre-competition point colonies, and another experiment in *B. terrestris* involving eggs experimentally transferred into egg-cups after been laid by individuals in microcolonies (Zanette et al. 2012) suggested that queen- and worker-laid eggs remain chemically distinct enough for discriminations to occur in introduction tests, indicating that cues of origin are not invariably lost in microcolonies. Second, chemical differences between eggs might have been decreased in the laboratory setting per se, i.e. (in the present case) in both full colonies and microcolonies, as has been shown to occur in *Pachycondyla* ants (van Zweden et al. 2009). Additionally, the commercial origin of our bees could conceivably have resulted in lower genetic diversity between our colonies than between wild *B. terrestris* colonies, which also could have decreased chemical differences. Again, however, any such effect did not prevent discrimination of natal and non-natal eggs in pre-competition point colonies in the current experiment, or *B. terrestris* workers discriminating between queen- and worker-laid eggs in the experiment of Zanette et al. (2012). Third, it is conceivable that the high degree of egg-eating in the current study was a product of using artificially constructed egg cells. However, workers were able to open these egg cells despite their artificial construction, and hence to make contact with the eggs inside them, as evidenced by the digital films showing that all filmed introduced egg cells were opened, even in cases in which all eggs remained at the end of 20 hours. In addition, rates of egg cell destruction were similar to those in the experiment of Zanette et al. (2012) that used egg cells constructed by *B. terrestris* queens and workers.

Overall, therefore, our results suggest that, in *B. terrestris*, egg eating can act as a general and effective form of policing against egg laying by workers, as, on average, 75.3% of introduced eggs were eaten. In addition, non-natal worker-laid eggs were more likely to be policed in pre-competition point colonies, in which, in nature, all worker-laid eggs would be from non-natal workers. These results have implications for understanding the evolution of both types of worker egg laying. In the case of egg laying by natal workers, it must first be noted that the prevalence of natal eggs is typically much higher than that of non-natal worker-laid eggs within colonies of *B. terrestris* and probably other *Bombus* species (see Introduction), and so worker policing in such species most probably originated as the policing of selfish workers, i.e. policing by other natal egg-laying workers (Wenseleers et al. 2005b; Zanette et al. 2012). Our study showed that the effectiveness of policing of natal eggs did not differ between the growth (pre-competition point) and post-competition point stages of the colony cycle (fig. 3). Therefore, workers in the pre-competition stage probably refrain from egg-laying through self-restraint, rather than from a greater risk of their eggs being policed, which is consistent with the idea that all natal workers share an interest in the colony achieving its mature colony size (Avila et al. 2019). In the case of egg laying by non-natal workers, the results suggest that, all else being equal, the socially parasitic strategy of these workers might be most successful in host colonies that have passed the competition point. However, a non-natal worker might still gain from egg laying within a host colony before the host colony's competition point, since this would increase the worker's chances of producing an ‘early male’ with a higher mating success (Lopez-Vaamonde et al. 2004).

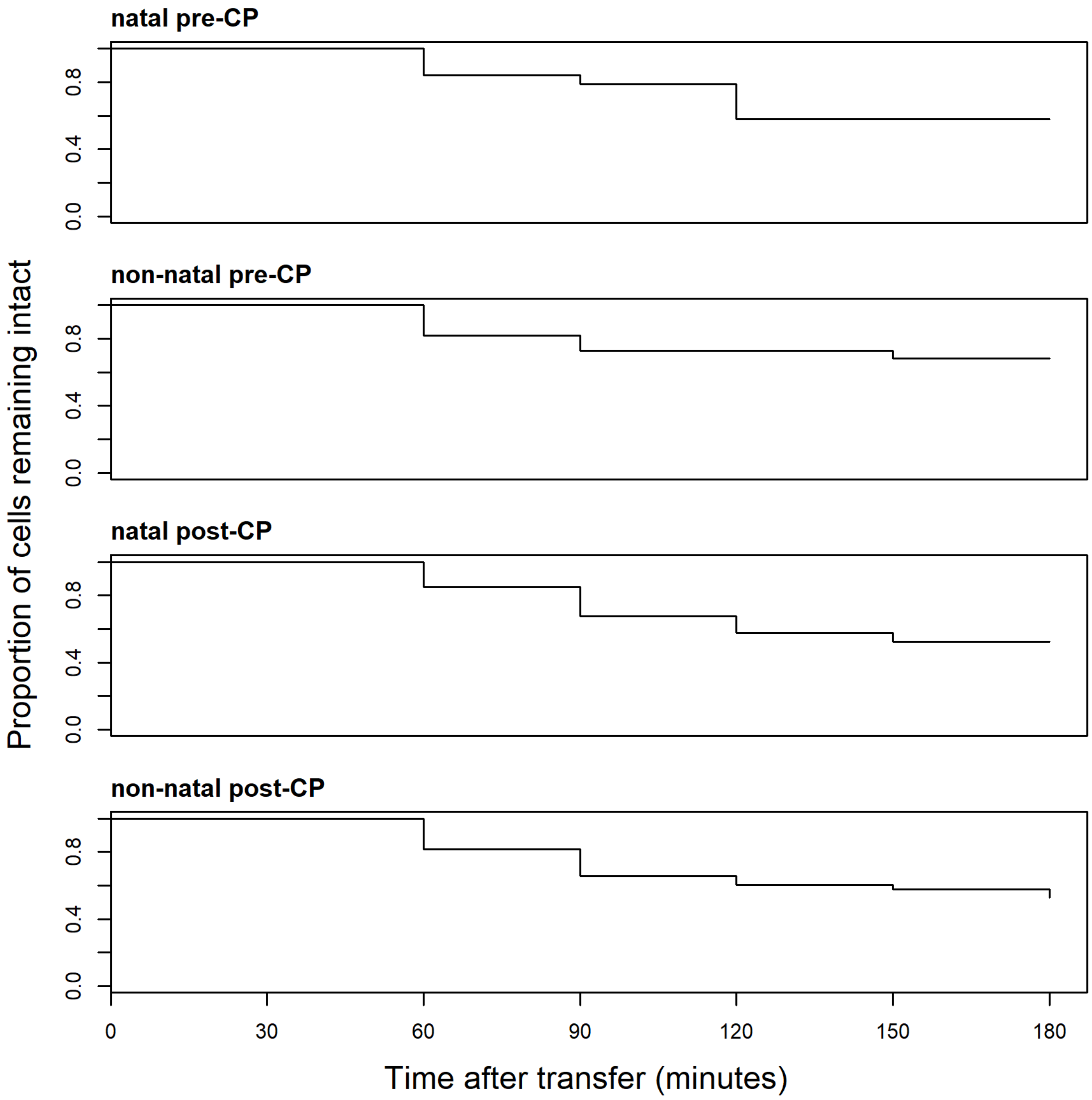
In conclusion, our results suggest that intraspecific social parasitism in bumblebees may have selected for context-dependent policing, whereby the eggs of non-natal workers are policed with greater effectiveness before the competition point. This is potentially surprising given the relatively low frequency of intraspecifically socially parasitic workers in bumblebees (see Introduction). However, drifting (and associated social parasitism) may have been ancestrally more common, as it is currently in some other bees (65% of workers drifting in *Melipona fasciculata*, Oliveira et al. 2021; 32% of nests containing unrelated workers in *Lasioglossum malachurum*, Soro et al. 2009) and in some wasps (up to 56% of workers drifting in *Polistes canadensis*, Sumner et al. 2007). Our data therefore suggest that intraspecific social parasitism may produce relatively strong selective effects, although the comparative importance of natal and non-natal worker reproduction in the evolution of worker policing deserves further investigation. A phylogenetic comparative analysis of the relative frequencies of each form of worker reproduction across *Bombus* species, and of any associated worker policing, would go far in addressing this issue.

## Figure legends

Fig. 1 The proportion of artificial egg cells introduced to *Bombus terrestris* colonies (n = 10 colonies) remaining intact (not destroyed) at 30, 60, 90, 120, 150 and 180 minutes after introduction. Natal pre-CP: natal worker eggs introduced to pre-competition point colonies (n = 19 egg cells); non-natal pre-CP: non-natal worker eggs introduced to pre-competition point colonies (n = 22 egg cells); natal post-CP: natal worker eggs introduced to post-competition point colonies (n = 40 egg cells); non-natal post-CP: non-natal worker eggs introduced to post-competition point colonies (n = 38 egg cells). Mean ± SD eggs per egg cell = 6.3 ± 1.5 eggs.

Fig. 2 The fate of artificial egg cells introduced to *Bombus terrestris* colonies (n = 10 colonies) 20 hours after introduction for cells containing eggs from natal or non-natal workers. Colony IDs (left-hand side of panels) with shared digits (1A, 1B etc.) identify paired colonies (5A and T4 were also paired). Each colony received 11-12 introduced egg cells over the time course of the experiment (experimental day), each egg cell being depicted by a separate circle. Filled circle = cell destroyed (opened with all eggs removed); open circle = cell not destroyed. Thick vertical lines indicate the date of the competition point for each colony. Asterisks indicate the date of queen death for each colony, where this occurred during the experiment.

Fig. 3 Mean proportions of eggs remaining in artificial egg cells introduced to *Bombus terrestris* colonies (n = 10 colonies) after 20 hours. Upper plots depict the mean proportion of eggs remaining for individual colonies as a function of egg origin and colony stage: natal or non-natal = natal or non-natal worker-laid eggs, respectively; pre-CP or post-CP = pre- or post-competition point receiving colonies, respectively. Sample sizes: natal, pre-CP, n = 19 egg cells; non-natal, pre-CP, n = 22 egg cells; natal, post-CP, n = 40 egg cells; non-natal, post-CP, n = 38 egg cells. Mean ± SD eggs per egg cell = 6.3 ± 1.5 eggs, 0–6 introductions per each of the four experimental conditions, per receiving colony. Error bar, ±1 SE. Horizontal lines with no error bar indicate no eggs remained in any introduction; blank plots indicate no introductions. Lower plots: violin plots showing the medians and distributions of proportions of remaining eggs. Black plots, pre-competition point colonies; grey plots, post-competition point colonies. Filled circles ± error bars depict median ± interquartile range.


natal pre-CP
non-natal pre-CP
natal post-CP
non-natal post-CP
Proportion of cells remaining intact
0.0
0.4
0.8
0
30
60
90
120
150
180
Time after transfer (minutes)

578

579 Fig. 1

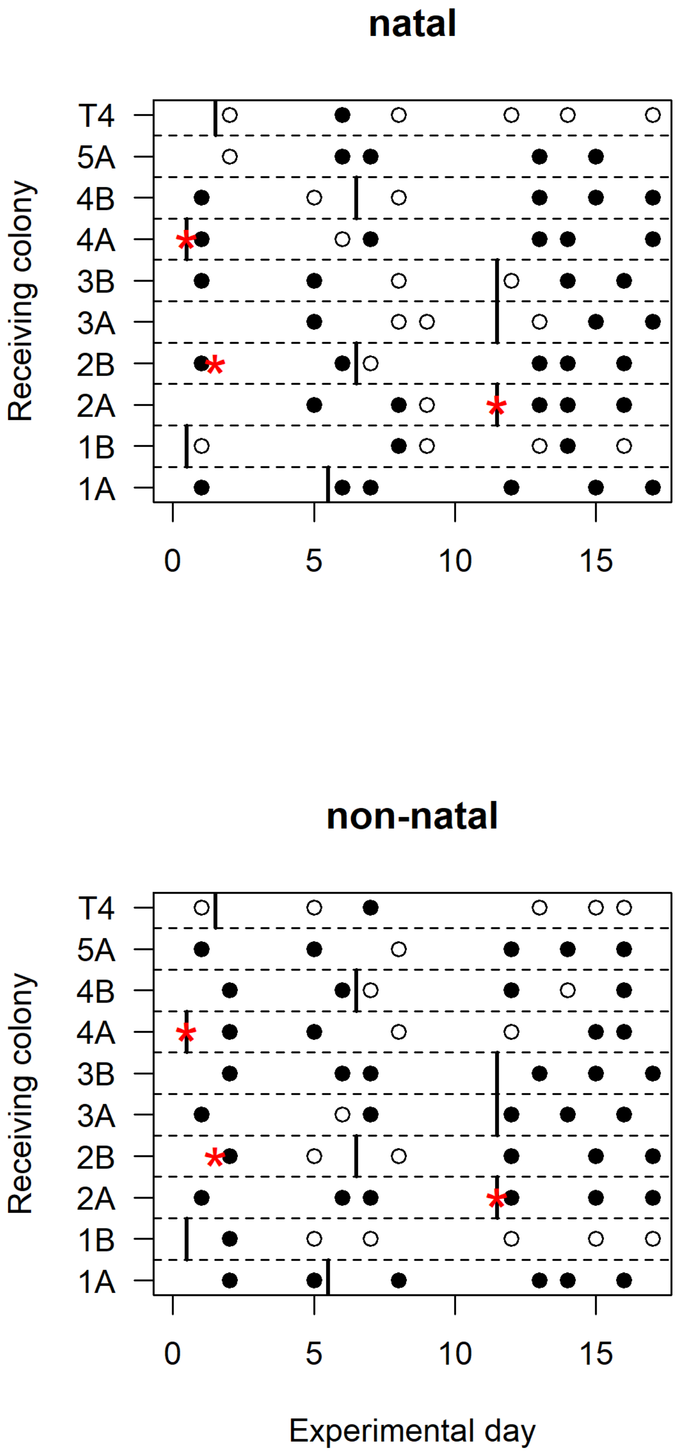

natal
Receiving colony
T4
5A
4B
4A
3B
3A
2B
2A
1B
1A
0
5
10
15
non-natal
Receiving colony
T4
5A
4B
4A
3B
3A
2B
2A
1B
1A
0
5
10
15
Experimental day

Fig. 2.

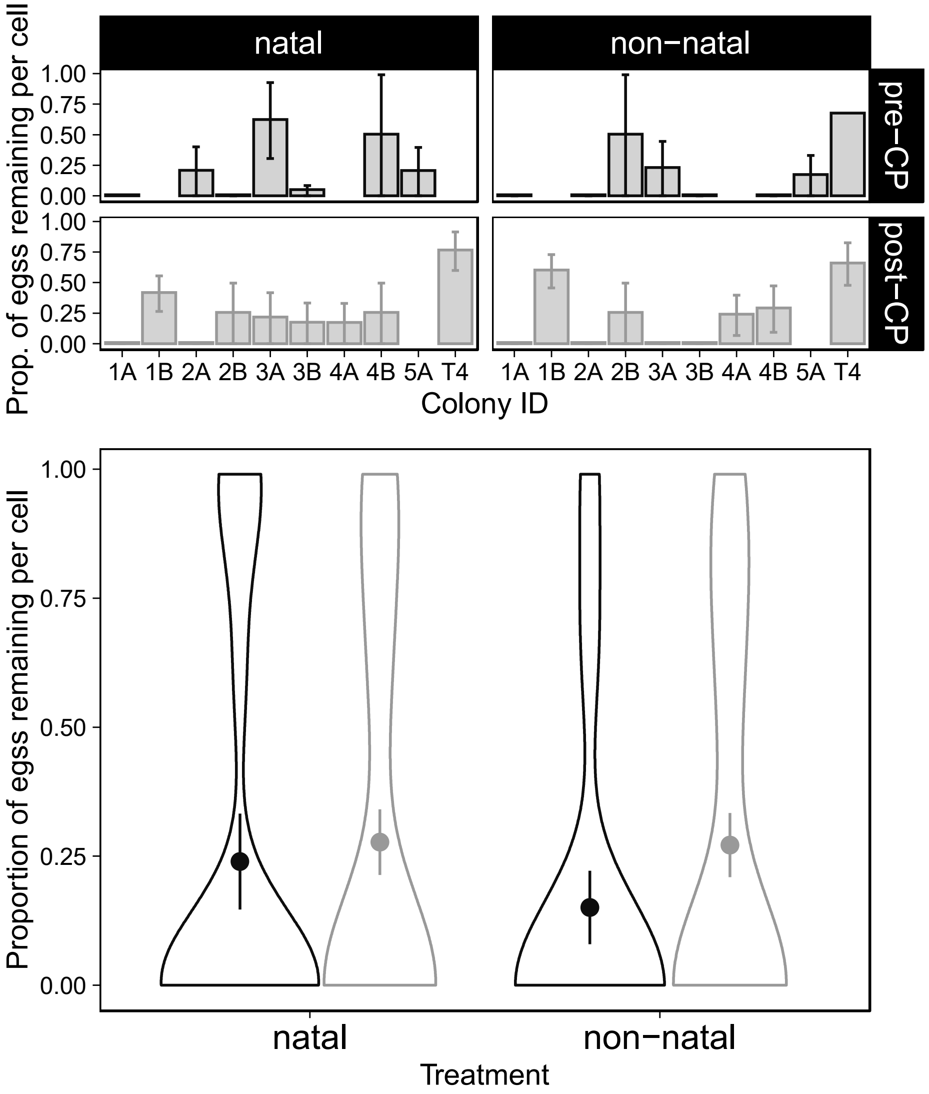


Fig. 3

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