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REPRODUCTIVE CONFLICT IN BUMBLEBEES AND THE EVOLUTION OF WORKER POLICING

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Worker policing (mutual repression of reproduction) in the eusocial Hymenoptera represents a leading example of how coercion can facilitate cooperation. The occurrence of worker policing in "primitively" eusocial species with low mating frequencies, which lack relatedness differences conducive to policing, suggests that separate factors may underlie the origin and maintenance of worker policing. We tested this hypothesis by investigating conflict over male parentage in the primitively eusocial, monandrous bumblebee, *Bombus terrestris*. Using observations, experiments, and microsatellite genotyping, we found that: (a) worker- but not queen-laid male eggs are nearly all eaten (by queens, reproductive, and nonreproductive workers) soon after being laid, so accounting for low observed frequencies of larval and adult worker-produced males; (b) queen- and worker-laid male eggs have equal viabilities; (c) workers discriminate between queen- and worker-laid eggs using cues on eggs and egg cells that almost certainly originate from queens. The cooccurrence in *B. terrestris* of these three key elements of "classical" worker policing as found in the highly eusocial, polyandrous honeybees provides novel support for the hypothesis that worker policing can originate in the absence of relatedness differences maintaining it. Worker policing in *B. terrestris* almost certainly arose via reproductive competition among workers, that is, as "selfish" policing.

KEY WORDS: *Bombus terrestris*, inclusive fitness theory, kin selection, social insect, worker reproduction.

Coercion represents a major factor promoting the evolution of cooperation (Frank 2003; Ratnieks and Wenseleers 2008; Bourke 2011). A leading example of coercion is worker policing in the haplodiploid eusocial Hymenoptera, whereby workers mutually prevent one another from producing male offspring (Ratnieks and Wenseleers 2008). Inclusive fitness theory predicts that, other things equal, worker policing occurs when workers are more closely related to queen-produced males than to worker-produced males (Starr 1984; Woyciechowski and Lomnicki 1987; Ratnieks 1988). Such relatedness differences arise from either polyandry

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(multiple mating by queens) or polygyny (multiple queens per colony), which modulate the queen–worker and worker–worker conflict over male parentage within colonies expected under monandry and monogyny (Bourke and Franks 1995; Crozier and Pamilo 1996). As inclusive fitness theory predicts, worker policing and associated patterns of male parentage occur as a function of relatedness differences in the highly eusocial, polyandrous honeybees, *Apis* (Ratnieks and Visscher 1989; Oldroyd et al. 2001), and, according to comparative analyses, across the eusocial Hymenoptera as a whole (Wenseleers and Ratnieks 2006).

Contrary to the general pattern, however, some "primitively" eusocial (i.e., having relatively low queen–worker dimorphism),

monandrous or weakly polyandrous Hymenoptera, which lack appropriate relatedness differences, exhibit worker policing (Wenseleers et al. 2005; Bonckaert et al. 2011). The mating system of these species is likely to reflect the ancestral state, given that high levels of polyandry are derived within the eusocial Hymenoptera (Hughes et al. 2008). Furthermore, some eusocial Hymenoptera with secondary parthenogenetic production of females (thelytoky), which again lack appropriate relatedness differences, also exhibit worker policing (Hartmann et al. 2003; Pirk et al. 2003). Cases like these have led to the hypothesis that factors aside from workers being more highly related to queen- than to worker-produced males contribute to the origin and maintenance of worker policing (Wenseleers et al. 2005; Pirk et al. 2007; Beekman and Oldroyd 2008; Ohtsuki and Tsuji 2009; Bonckaert et al. 2011). For example, it has been proposed that worker policing has evolved from reproductive competition among egglaying workers, that is from "selfish" or "corrupt" worker policing (Wenseleers et al. 2005; Bonckaert et al. 2011).

Worker policing as exhibited by honeybees, which we term "classical" worker policing, is in fact a syndrome characterized by several elements. Three key ones are as follows. First, workers (including nonreproductive workers) eat worker-laid male eggs, but not queen-laid male eggs, shortly after these eggs have been laid, heavily reducing the number of worker-produced males reaching adulthood (Ratnieks and Visscher 1989; Visscher 1989). Alternatively, workers may attack other ovary-activated workers (Visscher and Dukas 1995). Second, selective egg destruction occurs in the absence of reduced viability of worker-laid eggs, and so is not an aspect of colony "hygiene" (Ratnieks and Visscher 1989; Beekman and Oldroyd 2005). Third, worker discrimination of queen- and worker-laid male eggs occurs via workers' detection of chemical cues on eggs (Nanork et al. 2007). One or more of these elements occurs widely in other eusocial Hymenoptera exhibiting worker policing (e.g., Kikuta and Tsuji 1999; Endler et al. 2004; Bonckaert et al. 2008; Brunner and Heinze 2009). However, outside *Apis*, the cooccurrence of these three elements of classical worker policing has not been demonstrated.

Bumblebees (*Bombus* spp.) are primitively eusocial insects that are believed to resemble the primitively eusocial common ancestor of the two groups of highly eusocial corbiculate bees, the honeybees and stingless bees (Cardinal and Danforth 2011). *Bombus terrestris* is an annual bumblebee widely used as a model system in the study of social evolution (e.g., Baer and Schmid-Hempel 1999; Bloch and Hefetz 1999a; Lopez-Vaamonde et al. 2003, 2004, 2009; Duchateau et al. 2004; Alaux et al. 2006; Amsalem and Hefetz 2011). Colonies consist of a single queen and about 100–300 daughter workers (Alford 1975) and genetic studies demonstrate that queens are monandrous (Schmid-Hempel and Schmid-Hempel 2000; Lopez-Vaamonde et al. 2003, 2004;

Alaux et al. 2004). Workers are capable, via haplodiploidy, of producing male offspring from unfertilized, haploid eggs, but parentage analyses suggest that only a low proportion (about 5%) of adult males is worker-produced (Alaux et al. 2004; Lopez-Vaamonde et al. 2004). In the initial (ergonomic) phase of the colony cycle, queens produce only workers. Sexual production starts with the production of daughter queens or, following the "switch point" (defined as occurring when the queen lays her first haploid egg), haploid males (Duchateau and Velthuis 1988; Lopez-Vaamonde et al. 2009). Worker-produced males are reared only after the "competition point" (defined as occurring when the first worker-laid eggs appear), which generally falls after the switch point (Duchateau and Velthuis 1988; Lopez-Vaamonde et al. 2009). Because, unlike the case under polyandry, workers under a singly mated queen are more closely related to worker-produced males than to queen-produced males, inclusive fitness theory predicts that relatedness differences favoring worker policing are absent in *B. terrestris* (Trivers and Hare 1976; Ratnieks 1988).

In this study, our first aim was to quantify rates of egglaying and egg-eating by *B. terrestris* queens and workers, and resulting levels of worker-produced males, to determine the nature of conflict over male parentage in this species. Previous studies have reported worker aggression, egglaying, and egg-eating in *B. terrestris* (Van Honk et al. 1981; Van der Blom 1986; Van Doorn and Heringa 1986; Duchateau 1989; Bloch and Hefetz 1999b; Amsalem and Hefetz 2011), but these studies did not quantify the relative rates at which queens and workers lay and eat eggs. Because we found elements of both selfish and classical worker policing to be present, we then tested—with positive results—whether *B. terrestris* exhibits the three elements of classical worker policing described above. Our demonstration that these three elements cooccur in a primitively eusocial Hymenopteran, and moreover within a clade containing polyandrous, highly eusocial members that exhibit classical worker policing, provides new insight into the evolutionary origins of worker policing.

In total, we report four experiments using *B. terrestris*. In the first, we quantified the rates of egg laying and egg-eating by queens and workers and, in the same colonies, the frequency of worker-produced males among larvae and adults. In the second, we tested whether queen- and worker-laid male eggs differed in viability. In the third, we tested whether surface (chemical) cues permit worker discrimination of queen-laid from worker-laid eggs; because bumblebee eggs are laid within wax egg cells (with several eggs per egg cell), we also tested whether such cues reside on the surface of egg cells. Finally, in the fourth experiment, we investigated the possible marking of eggs and egg cells by queens by quantifying the relative roles of queens and workers in egg cell construction and sealing.

Methods

MEASUREMENT OF EGG-LAYING AND EGG-EATING RATES AND OF FREQUENCY OF LARVAL AND ADULT WORKER-PRODUCED MALES

A set of *B. terrestris terrestris* colonies (10 colonies) was obtained from a commercial supplier (Koppert Biological Systems, Haverhill, UK) in May 2007 (Table A1). Each colony was housed in a wooden nest box ($20 \times 30 \times 17$ cm high), with a clear Perspex lid, connected to an identical nest box to provide a feeding arena. Colonies were kept in standard conditions at $28°C$, 55–60% relative humidity, and constant darkness and fed with fresh pollen (Kiki Ltd., Aylsham, UK) and artificial nectar (Koppert Biological Systems). The queen and all workers were individually marked with numbered plastic discs glued to the thorax (Queen Marking Kit, Thorne Ltd., UK). Between 31 May 2007 (date acquired) and 17–27 July 2007 (depending on the colony), colonies were monitored every 2 days by a combination of observation and digital still photography to (a) census and mark newly eclosed workers, (b) census and remove newly eclosed sexuals (males and new queens), (c) determine the date of the switch point by retrospectively calculating from the date of first male eclosion (Lopez-Vaamonde et al. 2003), and (d) determine the date of the competition point. Newly eclosed sexuals were removed because, in nature, males and new queens leave the nest a few days after eclosion (Alford 1975). We analyzed only data on rates of egg laying and egg-eating (by queen and workers) collected after the competition point (competition phase), which was found to follow the switch point in all colonies (Table A1). Because the switch point involves a sharp transition to male egg laying (Duchateau et al. 2004), this meant that, over the period during which these data were collected, queens would have been laying almost exclusively male eggs. This was verified by the genotyping of late-instar larvae (see below), which showed that the modal percentage of males among larvae at the end of the observation period was 100% ($>$95% in seven of nine colonies sampled; Table A2). Nonetheless, laying by queens of a low frequency of diploid eggs during the observation period would have led to our estimate of the percentage of worker-laid male eggs escaping egg-eating (see Results) being a slight underestimate. All colonies remained in a queenright condition (i.e., with the colony queen alive) during the observation period, except for one (from which only data from its queenright period are reported; Table A1). All colonies were frozen 3–6 days after direct observations had ended, except for the colony whose queen died, which was kept alive for 13 days after the queen's death to permit collection of potentially worker-produced adult males (Tables A1, A2).

Rates of egg laying by colony queens were obtained by filming the brood area of five randomly selected colonies in a single bout of 6.5 h each (mean 9 days after the competition point).

Filming in this and all other experiments was conducted under red light using a Sony DCR-SR32 digital camcorder. Rates at which workers laid eggs and at which queens and workers ate eggs were determined by direct observation during 20–30 min bouts conducted between 1400 and 1900 every 1–2 days on each colony. Individual identities of all egg layers and egg eaters were recorded, as was, where known, the parentage of eggs eaten. Final rates of egg laying and egg-eating were then calculated as described below. Note that, despite egg cells harboring multiple eggs, all eggs within an egg cell can successfully develop as larvae, because the flexible cell walls expand as larvae grow until eventually each larva occupies its own, individual cell (Alford 1975); hence egg-eating genuinely reduces the productivity of each egg cell affected. Overall, colonies were directly observed for a mean of 6.3 h (range, 3–9 h) per colony over a mean period of 24 days (range, 5–36 days) after the competition point (Table A1).

To determine in detail the fate of newly laid queen and worker eggs, egg cells in which eggs were seen to be laid during the observation bouts were filmed following the end of the bout with digital camcorders mounted above nests. The time at which eggs were laid in the focal egg cells was noted and filming continued for up to 2 h from this time. Egg cells selected for filming were those in which the first egg-laying event (queen or worker) for that bout was recorded. However, because the camcorder's field of view was usually larger than one egg cell, egg-laying events in other egg cells were observed during the viewing of films, and data from these were also recorded and included in analyses. Overall, 7.4 ± 4.9 focal egg cells per colony were filmed over 1.52 ± 0.57 h per egg cell (Table A1). Any egg-laying and egg-eating events observed only in the films of the focal egg cells were added to the data from the direct observations, with rates of each type of event then being calculated over the total duration of direct observation and filming for each colony. Egg-laying and egg-eating events could be easily recognized on film because each was preceded by characteristic behaviors (placing of abdominal tip in egg cell and opening of egg cell, respectively) and each lasted a relatively long time (means of 2–4 min).

During both direct observations and filming of focal egg cells, the placement of a queen or worker's body during egg laying (68 of 187 events) or egg-eating (112 of 211 events) meant that the number of eggs laid or eaten could not always be directly observed. To estimate rates of egg laying and eating, we therefore multiplied the observed number of egg-laying (or egg-eating) events by the appropriate mean number of eggs laid (or eaten) calculated from those events during which counting eggs laid or eaten was possible. Data (mean $\pm$ SD) from such events showed that, per laying event, queens laid 5.7 ± 2.5 eggs (range 1–11, $n = 20$ events) and workers laid 2.7 ± 1.5 eggs (range 1–6, $n = 98$ events); and, per egg-eating event, queens ate 2.7 ± 1.4 eggs (range 1–6, $n = 31$ events) and workers ate 1.7 ± 1.0 eggs (range

1–4, $n = 68$ events). To estimate the net rate of worker-laid eggs surviving, we calculated the difference between the rate at which worker-laid eggs were laid and the total rate at which worker-laid eggs were eaten, assuming that no worker-laid eggs survived in colonies in which the rate at which they were eaten exceeded the rate at which they were laid in the sampling period.

Ovaries of a sample of workers were dissected to determine workers' reproductive status. All individually identifiable workers that had been observed eating eggs in each colony were dissected (one to five workers per colony). In addition, as controls, five to 17 individually identifiable workers, randomly selected from the set of workers in each colony that had not been observed egg-eating, were dissected (Table A1). Workers were defined as having activated ovaries if the average score of their ovarioles equaled 3 or more on a standard scale (Duchateau and Velthuis 1989) of 0 (inactive ovariole) to 5 (fully activated ovariole).

We used microsatellite genotyping to determine the frequency of worker-produced males among adult males and larvae. DNA was extracted from newly eclosed adult males (from a tarsus) and late-instar larvae (whole body) using a HotSHOT protocol (Truett et al. 2000). To obtain multilocus genotypes, six polymorphic unlinked microsatellite loci previously characterized in *B. terrestris* were used, *B10*, *B11*, *B96*, *B118*, *B121*, and *B124* (Estoup et al. 1993). Polymerase chain reactions (PCR) were carried out in 5 µl of a mixture containing 2.5 µl Qiagenmix, 1.0 µl primer multiplex (all six loci, quantities varied from 0.005 to 0.036 µM depending on the locus), 0.5 µl water, and 1.0 µl DNA ($\approx$ 50 ng). PCR multiplexes were performed in a Biometra TProfessional Standard Thermocycler as follows: an initial denaturation step at 95°C for 15 min, 30 cycles of 94°C for 30 s, 57°C for 90 s and 72°C for 60 s, and a final incubation at 60°C for 30 min. PCR products were run with a 500 LIZ^{TM} (Applied Biosystems, Carlsbad, CA) size standard on an ABI Prism 3730 automated DNA sequencer. Results were analyzed with GENEMAPPER (version 4.0; Applied Biosystems). CERVUS 3.0.3 (Marshall et al. 1998) was used to estimate standard intrapopulation sample genetic variability and the frequency of null alleles for each locus. GENEPOP 4.0 (Rousset 2008) was used to test for Hardy–Weinberg equilibrium. A positive and negative control was run with every PCR to ensure that amplification of alleles was consistent throughout the study, and to check for any contamination, respectively.

We genotyped samples of up to 48 newly eclosed adult males and 24 late-instar larvae from each colony (Table A2). Adult males for genotyping were selected from those eclosing 0–2 days before the termination date (date on which colonies were frozen). Because males take at least 24 days to develop from egg to adult (Bourke and Ratnieks 2001), this meant that in some colonies, there was a limited time window in which adult worker-produced males could have been collected (Table A1). It was for this reason that we also sampled late-instar larvae from each colony. These

were sampled from those present within colonies on the termination date. Therefore, given that male *B. terrestris* larvae pupate 18 days after being laid as eggs (Bourke and Ratnieks 2001), males in the sample of late-instar larvae would have included individuals developing from both queen- and worker-laid male eggs. Male larvae were identified from their multilocus genotypes because haploid males, unlike diploid females, appear homozygous at all loci (Lopez-Vaamonde et al. 2003). In eight of 10 colonies, a minimum of 20 larvae per colony were confirmed to be male larvae (Table A2).

To determine the mating type of each colony for the analysis of male parentage, we also genotyped all 10 colony queens and a sample of 6–23 daughter workers in each colony, including all workers used in the ovarian dissections (Table A1). We defined males (adult and larvae) as produced by workers if they had a paternal allele (i.e., allele of the queen's mate) at one or more loci (Lopez-Vaamonde et al. 2004). For each colony and each locus, an allele was considered to be paternal if it was found in every worker of that colony. The proportion of males produced by workers was corrected for nondetection of paternal alleles using the formula in Foster et al. (2001).

The number of alleles per locus varied from three to eight and expected heterozygosity ranged from 0.49 to 0.87. No significant deviations from Hardy–Weinberg equilibrium were found at any locus. Null allele frequencies were negative or close to zero for all loci, except for *B118*, in which a frequency of null alleles of 0.21 was detected. Nevertheless, paternal alleles at this locus were found in eight colonies, suggesting that there was little or no effect on the detection of worker-produced males. Within colonies, no more than three alleles were found at any one locus, confirming monandry. In addition, the frequency of adult males that were classified as worker-produced corresponded well with the frequency of male larvae that were so classified (Results; Table A2), supporting the accuracy of the analysis of male parentage.

RELATIVE VIABILITY OF QUEEN- AND WORKER-LAID MALE EGGS

A second set of *B. terrestris terrestris* colonies (nine colonies) was obtained from Koppert Biological Systems in June 2009 and maintained as in the previous experiment except that nest boxes were not connected to separate feeding arenas. Colonies (and their associated brood combs) were split with a wire mesh through which bees could not pass. The mean colony size (1 day before splitting) was 54.7 ± 24.8 workers. The queen and five workers were kept on one side of the mesh and all remaining workers on the other side. After a week, eggs were sampled by removal of one to five recently built, entire egg cells from both sides of the mesh over each of one to six different days per colony (between

24 July 2009 and 18 August 2009). From each egg cell, 1–16 eggs were successfully removed for scoring of their viability.

To score egg viability, we constructed "plates" of pure "beeswax" (i.e., from *Apis mellifera*) obtained from Thorne Ltd., UK. Each plate consisted of a thick disk of wax 50 mm in diameter in which we made 16 "wells" (about 0.5 cm in diameter) arranged in a grid pattern on the upper surface. A lipped lid of the same wax was made that could be placed on top of each plate. Eight eggs from the queenright side of the mesh and eight eggs from the queenless side of the mesh were randomly placed within the wells (one egg per well) of a given plate, having been transferred on clean cocktail sticks from the removed egg cells. The lid was fitted over each plate and the entire plate was placed in a small incubator (Brinsea, UK) set at 28°C and containing open pans of water to maintain high humidity (>95% RH). Wells were checked daily between 4 and 8 days after plates were placed in the incubators. Viability was scored as the proportion of eggs that hatched into living larvae in that interval, with scoring being conducted blindly with respect to source of the eggs.

To confirm the ploidy of eggs, unhatched eggs and larvae hatched from the eggs were collected from the incubated plates and genotyped at six microsatellite loci using the same methods as in the previous experiment. Only haploid eggs were included in the estimation of egg viability (Table A3). In addition, for haploid eggs from the queenright side of the mesh, we used parentage analyses (methods as in previous experiment) to ensure that only genuinely queen-produced eggs were included in the estimation of the viability of the queen's male eggs. Only worker-produced eggs from the queenless side of the mesh were included in the estimation of the viability of workers' male eggs.

WORKER DISCRIMINATION OF QUEEN- AND WORKER-LAID EGGS

A third set of *B. terrestris terrestris* colonies (18 colonies) was obtained from Koppert Biological Systems in July 2010 and maintained as in the previous experiment except that bees were fed with defrosted fresh pollen (Koppert Biological Systems). Colonies were monitored daily to record the competition point, whereupon eight were randomly designated as receiver colonies and 10 as source colonies. All colonies had passed their switch point (retrospectively calculated from the date of first male eclosion) and 15 of 18 had passed their competition point (seven of eight receiver and eight of 10 source colonies) by the date of each colony's first trial, so queens in source colonies would have been laying all or mainly male eggs at the time of the trials. From each source colony, the queen and three sets of three workers were taken and placed in a small plastic box each. The four boxes were each provided with three to five larval cells from the natal source colony and regularly provisioned with pollen and artificial nectar. The

isolated queens and worker groups constructed egg cells and laid eggs within them, and these were used as test eggs and egg cells for the experiment.

By transferring eggs between egg cells from the isolated queen and worker groups in a cross-fostering design, we created the following four types of experimental egg cell: (I) queen-built egg cell containing queen-laid eggs; (II) queen-built egg cell containing worker-laid eggs; (III) worker-built egg cell containing queen-laid eggs; (IV) worker-built egg cell containing worker-laid eggs. In addition, as a control for our handling of egg cells and eggs, we created a fifth type (V) of experimental egg cell by removing egg cells from receiver colonies, extracting their eggs, and then replacing the same eggs. All experimental egg cells were created by carefully extracting eggs from egg cells and transferring them (with a cocktail stick) to egg cells of the appropriate type that had been emptied. The experimenter then resealed each egg cell. Cross-contamination was avoided by the experimenter wearing clean Latex gloves, handling egg cells with forceps cleaned with ethanol and rinsed with water between each use, and transferring each set of eggs with fresh cocktail sticks in sterile Petri dishes. In Types I–IV, the number of eggs transferred into each egg cell was recorded in each case (mean five eggs, range two to eight eggs). In these types, the queen or workers that had built the egg cell were non-nestmates of the queen or workers providing the eggs (e.g., in Type I, queens that had built the egg cells were not from the same source colony as queens that had laid the eggs).

Within 1 h of each being created, the experimental egg cells were introduced one-by-one into non-nestmate (egg cell Types I–IV) or nestmate (Type V) receiver colonies. All receiver colonies were queenright. The egg cell was placed on the main body of the brood comb and its exact position was recorded. At 20 h after introduction, an experimenter, who was blind to egg-cell type, recorded whether the egg cell had been destroyed or not (in "destroyed" egg cells, the egg cell had been opened and all eggs inside had disappeared). The experimenter also removed any introduced egg cell that had not been destroyed and counted the number of eggs remaining inside it. This was repeated until each receiver colony had received (in random sequence, at intervals >24 h) up to three experimental egg cells of each type. In total, 118 experimental egg cells containing 661 eggs were used in the experiment.

To confirm that any receiver-colony treatment of introduced egg cells was carried out by workers not queens, we digitally filmed 26 introduced egg cells (randomized across each type) for the first 3 h after introduction. In all cases, only workers attended or opened the egg cells or ate eggs inside them (*n* = 26, 15, and 9 egg cells, respectively). Moreover, neither queens nor workers in receiver colonies laid new eggs within the introduced egg cells.

RELATIVE ROLES OF QUEENS AND WORKERS IN EGG-CELL CONSTRUCTION AND SEALING

A fourth and final set of *B. terrestris terrestris* colonies (10 colonies) was obtained from Syngenta Bioline Bees B.V., Weert, The Netherlands, in January 2010 and maintained as in the previous experiment. Starting 11.8 ± 7.3 days after the switch point (retrospectively calculated from the date of first male eclosion), unmanipulated colonies were digitally filmed for 3–4 h at weekly intervals until the natural death of the queen (filming lasted 50.1 ± 18.8 days). We analyzed a total of 72 h of film from the period 1 week after filming started (early period) and 1 week before each queen's death (late period). Because of queen deaths in two colonies, early-period data came from all 10 colonies and late-period data from eight of the colonies. The total numbers of egg cell-building events filmed over the sampled period of 72 h were 10 (early period, queen laid in egg cell), 12 (early period, worker laid in egg cell), 3 (late period, queen laid in egg cell), and 15 (late period, worker laid in egg cell). From the film data, for each egg cell, we (a) calculated the amount of time the queen or workers spent on the egg cell, measured from 1 h before to 1 h after eggs were laid in the egg cell, (b) recorded which party laid in the egg cell, and (c) recorded which party sealed (capped) the egg cell with wax. Because data from the early and late periods did not differ significantly (GLMM, $\chi^2 = 1.57$, df $= 1$, $P = 0.210$), they were pooled in further analyses.

STATISTICAL ANALYSES

Results were analyzed with R (R Development Core Team 2011). In the first and second experiments, two-sample tests were carried out using nonparametric tests (Wilcoxon signed rank or rank-sum tests) because of nonnormal distributions. In the third experi-
ment, results were analyzed using a Generalized Linear Mixed Model (GLMM), with either the proportion of egg cells or the proportion of eggs surviving after 20 h as the response variable, introduced egg-cell type as a fixed effect and receiver colony as a random effect. Within experiments, results are presented from all experimental colonies unless otherwise stated. All means are presented $\pm$ 1 SD unless otherwise stated.

Results

MEASUREMENT OF EGG-LAYING AND EGG-EATING RATES AND OF FREQUENCY OF LARVAL AND ADULT WORKER-PRODUCED MALES

After the competition point, *B. terrestris* queens laid 1.18 ± 0.70 eggs per colony per hour (these eggs being almost exclusively male; see Methods) and workers collectively laid 2.62 ± 1.22 eggs per colony per hour (Table 1). Therefore, on average, workers accounted for 69% of all eggs laid during the competition phase. All egg-eating, whether by queens or workers ($n = 83$ and 128 events, respectively), occurred only during the competition phase and was from egg cells known to contain only worker-laid eggs. Hence, queen-laid eggs were never or only rarely eaten but worker-laid eggs were eaten by both the queen and workers. Queens ate 1.41 ± 1.00 eggs per colony per hour and workers collectively ate 1.45 ± 0.67 eggs per colony per hour (Table 1). From these data, net rates of egg production (rates of egg survivorship) were estimated as 1.18 ± 0.70 eggs per colony per hour for queen-laid eggs (i.e., all queen-laid eggs survived) and 0.18 ± 0.32 eggs per colony per hour for worker-laid eggs (Table 1). Therefore, only an estimated 6.9% (0.18/2.62) of worker-laid eggs escaped being eaten and the expected parental contribution of workers to

Table 1. Data on levels of egg laying and egg-eating in *Bombus terrestris* colonies. All data came from the competition phase of queenright colonies. Total $n = 187$ egg-laying events and 211 egg-eating events over 133.5 h across 10 colonies, from both direct observations and focal egg-cell filming. Queen-laid eggs were almost exclusively male eggs and all eggs eaten were worker-produced eggs. See also Table A1.

Colony no.	No. of eggs laid per hour		No. of eggs eaten per hour		Net rate of worker-laid eggs surviving (no. of eggs per hour)
	By queen	By workers	By queen	By workers	
1	2.25	1.68	0.75	1.25	0.00
2	1.50	2.62	1.12	1.33	0.17
3	0.75	3.00	1.12	1.25	0.63
4	0.35	3.17	1.58	1.53	0.06
5	1.81	3.61	0.45	2.26	0.90
6	0.00	0.30	0.30	0.00	0.00
7	1.12	4.87	3.75	1.37	0.00
8	1.00	1.87	1.12	1.16	0.00
9	1.89	2.37	1.74	2.21	0.00
10	1.09	2.73	2.18	2.18	0.00
Mean $\pm$ SD	1.18 ± 0.70	2.62 ± 1.22	1.41 ± 1.00	1.45 ± 0.67	0.18 ± 0.32

the pool of larval and adult males, assuming no further differential mortality, was 13% (0.18/(0.18 + 1.18)).

The queen, reproductive workers (defined as workers observed laying eggs), and nonreproductive workers (defined as workers not observed laying eggs) all ate eggs, their rates of (worker-laid) egg-eating being 1.41 ± 1.00 (as above), 0.79 ± 0.66, and 0.66 ± 0.59 eggs per colony per hour, respectively. Hence, overall, queens, reproductive workers, and nonreproductive workers ate 49.3%, 27.6%, and 23.1%, respectively, of eggs eaten. Reproductive workers had higher mean rates of egg-eating per capita than nonreproductive workers (0.36 ± 0.43 vs. 0.14 ± 0.09 eggs per worker per hour, respectively), although this difference was not significant (Wilcoxon signed rank test: $V = 14$, df $= 10$, $P = 0.343$). However, the ovarian dissections showed that 41 of 105 "nonreproductive" workers (39%) had activated ovaries, suggesting that this worker class included a substantial proportion of reproductively active workers. In addition, ovarian dissections showed that workers observed egg-eating were significantly more likely to have activated ovaries (17 of 22) than workers not observed egg-eating (36 of 96; $\chi^2 = 9.9$, df $= 1$, $P = 0.002$). This supported the idea that egg-eating was indeed positively linked to worker reproduction. Nevertheless, some egg-eating workers (five of 22, i.e., 23%) had inactive ovaries, showing that egg-eating was not exclusively performed by reproductively active workers.

Filming of focal egg cells revealed that only 4% of worker-laid eggs remained 2 h after being laid, with 67% of worker-laid eggs having been eaten within 20 min of being laid (Fig. 1). The mean intervals between focal eggs being laid and their being eaten by queens and reproductive workers (18.7 ± 20.4 min and 24.9 ± 23.6 min, respectively) were significantly greater than the corresponding mean interval for eggs eaten by nonreproductive workers (7.7 ± 6.8 min) (Wilcoxon rank-sum tests: queens vs. reproductive workers, $W = 795$, df $= 54$, $P = 0.294$; reproductive vs. nonreproductive workers, $W = 884$, df $= 35$, $P < 0.001$). Hence, worker-laid eggs were eaten (policed) shortly after being laid, and queens and reproductive workers were more persistent policers than nonreproductive workers.

Microsatellite genotyping showed that the mean frequency of worker-produced males was 0.10 ± 0.17 among late-instar larvae and 0.09 ± 0.14 among adult males (Table A2). These frequencies were very close to those expected on the basis of the relative overall rates at which queens and workers laid and ate eggs (0.13, see above). In addition, within colonies, the overall frequency of worker-produced males (from combined larval and adult data) was not significantly different from the estimated frequency of worker-laid eggs that escaped policing (Wilcoxon signed rank test: $V = 7$, df $= 8$, $P = 0.892$). These findings suggested that the observed frequency of adult worker-produced males in *B. terrestris* can be entirely accounted for by policing of worker-laid eggs.
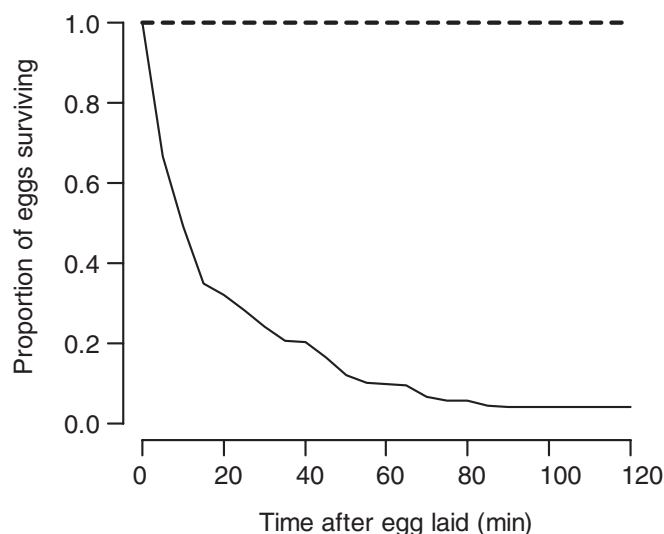

Figure 1. Survivorship within unmanipulated *Bombus terrestris* colonies of eggs in focal egg cells known to contain either queen-laid eggs (dashed line) or worker-laid eggs (solid line) and filmed for up to 2 h after they were laid. Decrease in proportion of worker-laid eggs stemmed from egg-eating by the queen, reproductive workers, or nonreproductive workers. Total sample sizes were, for queen-laid eggs, 35 eggs in six events filmed for 60 min each plus 43 eggs in seven events filmed for 120 min each, and, for worker-laid eggs, 239 eggs in 137 events filmed for up to 120 min each. An egg-eating "event" was as defined in Methods. See also Table A1.

RELATIVE VIABILITY OF QUEEN- AND WORKER-LAID MALE EGGS

Viabilities of incubated male eggs laid by *B. terrestris* queens and workers were not significantly different (queen-laid male eggs, viability $= 0.61 \pm 0.36$, $n = 71$ eggs from nine colonies; worker-laid male eggs, viability $= 0.58 \pm 0.22$, $n = 174$ eggs from same nine colonies; Table A3) (Wilcoxon signed rank test: $V = 23$, df $= 9$, $P = 1.0$). Therefore, worker-laid male eggs were not policed through having lower viabilities than queen-laid male eggs.

WORKER DISCRIMINATION OF QUEEN- AND WORKER-LAID EGGS

B. terrestris workers destroyed significantly more egg cells (100%), including all eggs inside them, when presented with worker-built egg cells containing worker-laid eggs (Type IV) than when presented with egg cells of any of Types I–III (Fig. 2A). Across Types I–III, there were no significant differences among either the percentages of egg cells destroyed (Fig. 2A) or, within surviving egg cells, among the percentages of eggs destroyed (Fig. 2B). Results from the control egg cells (Type V) showed that significantly fewer (nine of 27, 33%) egg cells were destroyed after 20 h relative to the percentages of egg cells destroyed within
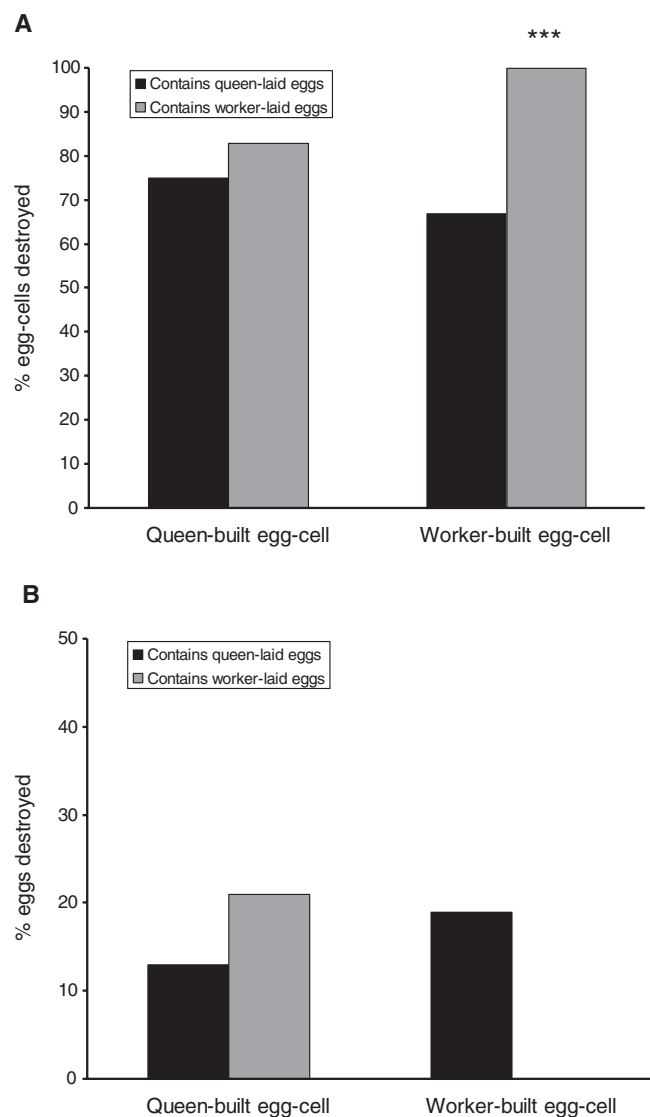
A

B

Figure 2. Percentage of experimental *Bombus terrestris* egg cells and eggs destroyed by 20 h after introduction into receiver colonies. (A) Percentage of egg cells destroyed. Sample sizes (no. of egg cells destroyed/no. introduced) were: 15/20 (Type I, queen-built egg cell containing queen-laid eggs), 19/23 (Type II, queen-built egg cells containing worker-laid eggs), 16/24 (Type III, worker-built egg cells containing queen-laid eggs) and 24/24 (Type IV, worker-built egg cells containing worker-laid eggs). Percentage destroyed in Type IV differed significantly (***) from the percentages in Types I–III, which did not differ significantly from one another (GLMM: Type IV vs. Types I–III: $P = 0.001$; Types I–III vs. Types I–III: all $P > 0.05$). (B) For egg cells surviving after 20 h, percentage of eggs destroyed within them. Sample sizes (no. of eggs destroyed/no. introduced): 4/32 (Type I), 5/24 (Type II), and 9/47 (Type III). No Type IV egg cells were present in this sample because all were destroyed by 20 h. None of the percentages for Types I–III differed significantly from one another (GLMM: all $P > 0.05$).

Types I–III and IV ($P = 0.048$ and $P < 0.001$, respectively). This suggested that the extent of egg destruction in Types I–IV stemmed partly from the receiving workers' response to the experimental handling of the egg cells and eggs. In addition, some of the effect was likely to have stemmed from egg cells and eggs deriving from non-nestmates of the receiver colonies. However, the significant difference between the proportion of egg cells destroyed between Types I–III and IV (Fig. 2A) must have stemmed from the factor of egg-cell type itself, because all egg cells and eggs in these types received the same experimental handling and all were derived from non-nestmates of receiver colonies. Therefore, the results suggested that surface cues produced by the queen are present on both queen-built egg cells and queen-laid eggs and that the absence of both these cues permits workers to discriminate against worker-laid eggs whereas the presence of any one of them causes workers to treat eggs equally. Given that our experiment used eggs and egg cells from queens that were not nestmates of receiver colonies, the cue appears not to be colony-specific.

RELATIVE ROLES OF QUEENS AND WORKERS IN EGG-CELL CONSTRUCTION AND SEALING

In unmanipulated *B. terrestris* colonies in the competition phase, each party (queen or worker) spent significantly more time on egg cells in which it laid eggs than on those in which the other party laid eggs (Fig. 3). In addition, in egg cells in which the queen laid the eggs, the queen sealed the egg cell in 92% (12 of 13) of cases, and, in egg cells in which workers laid the eggs, workers sealed the egg cell in 100% (27 of 27) of cases. Therefore, the party that laid eggs in an egg cell was significantly likely to be the party that sealed the egg cell (Fisher's exact test, $P < 0.001$). Together with the results of the previous experiment, these findings suggest that, during egg laying and cell sealing, queens deposit a chemical on eggs and egg cells that is the surface cue used by workers to discriminate between queen- and worker-laid eggs. Moreover, because worker-laid eggs generally occur within worker-built egg cells (Fig. 3), the egg/egg cell combination permitting worker discrimination of worker-laid eggs (Fig. 2A) is the one workers would usually encounter in unmanipulated nests.

Discussion

We found that *B. terrestris* exhibits three key elements of classical worker policing despite lacking a colony kin structure in which workers are more related to queen- than to worker-produced males. Specifically, our findings show that in *B. terrestris*: (a) worker- but not queen-laid male eggs are nearly all eaten (by queens, reproductive, and nonreproductive workers) soon after being laid, and that this explains the low frequency of larval and adult worker-produced males observed in the same colonies
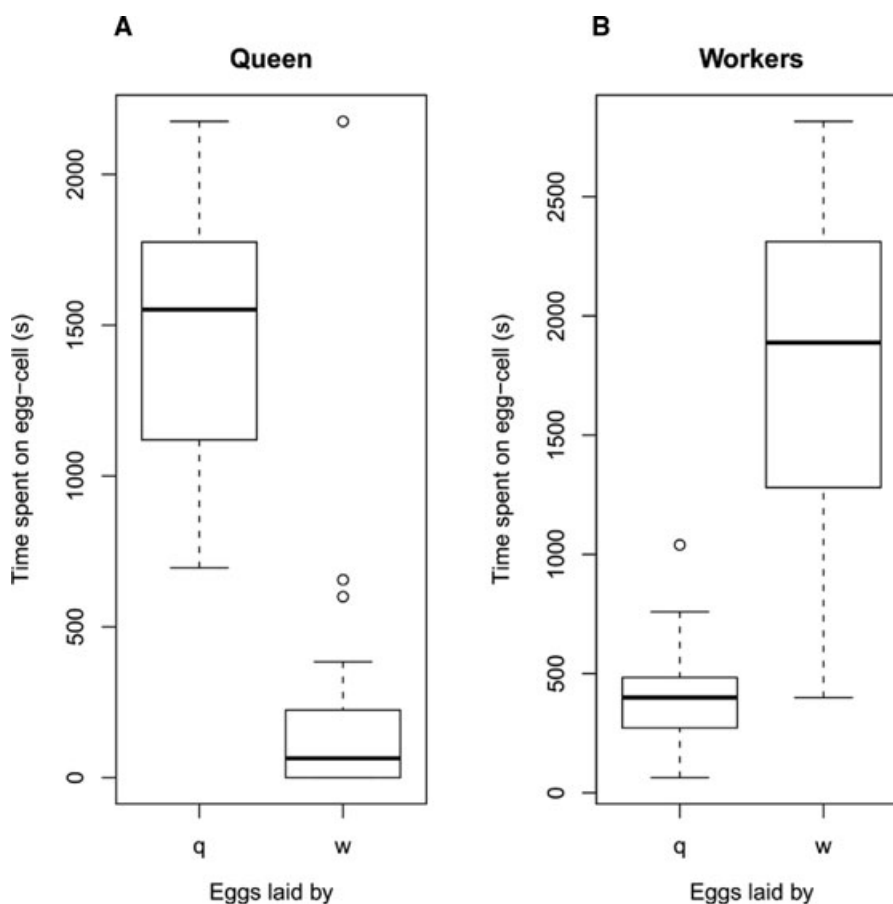
Figure 3. Time spent by *Bombus terrestris* queens and workers on egg cells as a function of which party laid eggs in the egg cell. (A) Queen's time spent on egg cells in which queen (q) or workers (w) laid eggs. (B) Workers' time spent on egg cells in which queen (q) or workers (w) laid eggs. Boxplots depict median values (bold bar) and interquartile range (upper and lower borders), with whiskers extending to 1.5 times the interquartile range and outlying points plotted as open circles. Post hoc Tukey contrasts: queen versus worker time when eggs laid by queen: $Z = 202$, $P < 0.001$; Queen versus worker time when eggs laid by workers: $Z = 140$, $P < 0.001$; $n = 13$ egg cells in which queen laid eggs and 27 egg cells in which workers laid eggs.

(this study) and, by implication, the low frequency of worker-produced adult males observed in previous parentage analyses of *B. terrestris* males (Alaux et al. 2004; Lopez-Vaamonde et al. 2004); (b) queen- and worker-laid male eggs have equal viabilities, showing that selective egg-eating does not occur for "hygienic" reasons; (c) workers discriminate between queen- and worker-laid eggs using surface cues on eggs and egg cells that are likely to be chemical cues deposited by the queen. Other primitively eusocial Hymenoptera with low mating frequencies resemble *B. terrestris* in exhibiting one or two of these traits, but in none of them has the cooccurrence of all three elements of classical worker policing been demonstrated (e.g., Saigo and Tsuchida 2004; Wenseleers et al. 2005; Bonckaert et al. 2011).

These findings provide strong support for the hypothesis that the worker policing syndrome can originate in the absence of, and likely before, the evolution of relatedness differences conducive to its maintenance (see Introduction). Notwithstanding the derived

nature of polyandry in the eusocial Hymenoptera, including the corbiculate bees (Hughes et al. 2008), *B. terrestris* arguably has an ancestry of polyandry because it belongs to the subgenus *Bombus* sensu stricto, whose sister subgenus, *Pyrobombus* (Cameron et al. 2007), exhibits polyandry (Payne et al. 2003; Huth-Schwarz et al. 2011). In addition, it is possible that the evolution of a male mating plug has secondarily imposed monandry on *B. terrestris* queens (Sauter et al. 2001). However, polyandry appears derived even within *Pyrobombus* (Schmid-Hempel and Schmid-Hempel 2000; Payne et al. 2003) and, in polyandrous *Pyrobombus*, effective mating frequencies are less than 2 (Payne et al. 2003; Huth-Schwarz et al. 2011) and hence below the level selecting for worker policing (Ratnieks 1988). Therefore, worker policing in *B. terrestris* is unlikely to stem from ancestral polyandry and the conclusion remains that factors other than workers being more related to queen- than to worker-produced males were almost certainly responsible for the origin of worker policing in this species.

Our findings also support one of the main factors that has been suggested to lead to worker policing in the absence of appropriate relatedness differences, namely competition among reproductive workers or selfish worker policing, as also detected in some wasps (Wenseleers et al. 2005; Bonckaert et al. 2011) and ants (Stroeymeyt et al. 2007). Under a singly mated queen, workers in eusocial Hymenoptera are related to males in the descending order, sons ($r = 0.5$), males produced by other workers ($r = 0.375$), and males produced by the queen ($r = 0.25$). According to the concept of selfish worker policing, workers prevent other workers from reproducing to maximize their direct fitness gains (i.e., through favoring the production of sons). In the present study, this explanation was supported by egg-laying workers tending to eat more eggs per capita and eating significantly older eggs, and by egg-eating workers being significantly more likely to have active ovaries. At first sight, selfish worker policing does not explain why workers with inactive ovaries also ate workers' eggs. However, the distinction between reproductive and nonreproductive workers in *B. terrestris* may not be absolute, in that, conceivably, such workers were priming themselves to activate their ovaries in future.

Three other factors that have been proposed to lead to worker policing in the absence of appropriate relatedness differences are unlikely to operate in *B. terrestris*. Worker policing could have been selected because it increases overall colony efficiency (Ratnieks 1988). But this factor is probably not a key one because an experimental measure of the costs of worker reproduction in *B. terrestris* found them to be absent or low (Lopez-Vaamonde et al. 2003). It has been hypothesized that worker policing evolved to prevent worker reproduction disrupting the ergonomic phase of eusocial colonies (Ohtsuki and Tsuji 2009). However, in *B. terrestris*, the existence of the competition point makes this factor unlikely because it means that workers never lay eggs during the ergonomic phase, which suggests they are then reproductively self-restrained (Bourke and Ratnieks 2001). Arguably, the threat of policing deters workers from egg laying in the ergonomic phase. But this is probably not the case, because conspecific workers from unrelated colonies ("drifters") do sometimes lay eggs before their host colony's competition point in *B. terrestris* (Lopez-Vaamonde et al. 2004) and their eggs are not immune from policing (Zanette et al., unpubl. data). Worker policing could also have arisen precisely to serve as a defense against egg laying by conspecific non-nestmate workers (Pirk et al. 2007; Beekman and Oldroyd 2008), which occurs in several eusocial bees as well as *B. terrestris* (Lopez-Vaamonde et al. 2004; Nanork et al. 2005; Beekman and Oldroyd 2008; Takahashi et al. 2010). However, such intraspecific worker social parasitism does not appear to be sufficiently frequent in wild *B. terrestris* (Zanette et al., unpubl. data) to have generated selection for a universal favoring by workers of queen- over worker-laid eggs. This again suggests that this

explanation is unlikely, although comparative studies would be required to exclude the possibility that intraspecific worker social parasitism was ancestrally more frequent.

An additional finding of the present study was that, in *B. terrestris*, the queen polices workers' male eggs at a high rate, as other authors have suggested (Duchateau 1989; Velthuis et al. 2002). Queen policing is predicted by inclusive fitness theory, because, under both monandry and polyandry, the queen is more closely related to her sons ($r = 0.5$) than to those of workers ($r = 0.25$). The fact that, through extensive egg policing, the queen severely reduces the success of worker reproduction provides an evolutionary rationale for worker matricide of queens, which is sometimes observed during the competition phase in *B. terrestris* (Bourke 1994; Bloch and Hefetz 1999b).

A result in the present study not explained by the concept of selfish worker policing was the finding that both reproductive and nonreproductive *B. terrestris* workers avoided eating less related, queen-laid male eggs. Such avoidance may not be total because reports of workers eating queen-laid eggs exist in *B. terrestris* (e.g., Van der Blom 1986; Duchateau 1989) and other *Bombus* species (Alford 1975). However, in the present study, *B. terrestris* workers either never ate queen-laid male eggs or did so at very low rates. It is possible that, ancestrally, workers ate queen-laid male eggs more frequently, but, in the absence of similar studies, it is unclear whether this is the case. Regardless, the avoidance by egg-eating workers of queen-laid male eggs, which may be shared by *Dolichovespula* wasps (Wenseleers et al. 2005; Bonckaert et al. 2011), is a principal element of classical worker policing. Its occurrence in *B. terrestris* therefore links the policing syndrome shown by *B. terrestris* with classical worker policing, which otherwise differs in that, in classical worker policing, it is almost exclusively nonreproductive workers that police worker-laid eggs.

A possible reason for workers avoiding eating queen-laid male eggs is suggested by our finding indicating that queens mark egg cells and eggs using queen-specific chemical cues. Such chemical cues may be linked to chemical differences between queen- and worker-laid eggs (Ayasse et al. 1999) and to chemical signals of fecundity in queens and workers in *B. terrestris* (Sramkova et al. 2008; Amsalem et al. 2009). For example, as studies of other eusocial Hymenoptera have suggested (Endler et al. 2006; Peeters and Liebig 2009), workers may use cues on queen-laid eggs to monitor queen fertility and avoid eating queen-laid eggs, including male ones, if they thereby assess queens as highly productive. However, from a comparison of relatedness coefficients alone, *B. terrestris* queens would have to be 1.5 times (0.375/0.25) more productive of males than workers for workers to favor queen- over worker-produced males, yet our data showed that in fact workers in the competition phase collectively laid more eggs than queens. Hence, why *B. terrestris* workers eat few

or no queen-laid male eggs remains to be fully determined. How worker policing as exhibited in *B. terrestris* might become modulated into the classical form also remains to be explored. *Bombus* species vary widely in the frequency of adult worker-produced males (e.g., Paxton et al. 2001; Brown et al. 2003; Alaux et al. 2004; Lopez-Vaamonde et al. 2004; Takahashi et al. 2008, 2010; Huth-Schwarz et al. 2011) and in levels of behavioral expression of conflict over male parentage (reviewed in Wenseleers and Ratnieks 2006). Given phylogenies exist for both *Bombus* and corbiculate bees as a whole (Cameron et al. 2007; Cardinal and Danforth 2011), studies of this issue in a comparative context now seem feasible.

LITERATURE CITED

Alaux, C., F. Savarit, P. Jaisson, and A. Hefetz. 2004. Does the queen win it all? Queen-worker conflict over male production in the bumblebee, *Bombus terrestris*. Naturwissenschaften 91:400–403.

Alaux, C., P. Jaisson, and A. Hefetz. 2006. Regulation of worker reproduction in bumblebees (*Bombus terrestris*): workers eavesdrop on a queen signal. Behav. Ecol. Sociobiol. 60:439–446.

Alford, D. V. 1975. Bumblebees. Davis-Poynter, London.

Amsalem, E., and A. Hefetz. 2011. The effect of group size on the interplay between dominance and reproduction in *Bombus terrestris*. PLoS One 6:e18238.

Amsalem, E., R. Twele, W. Francke, and A. Hefetz. 2009. Reproductive competition in the bumble-bee *Bombus terrestris*: do workers advertise sterility? Proc. R. Soc. B 276:1295–1304.

Ayasse, M., J. Birnbaum, J. Tengö, A. Van Doorn, T. Taghizadeh, and W. Francke. 1999. Caste- and colony-specific chemical signals on eggs of the bumble bee, *Bombus terrestris* L. (Hymenoptera: Apidae). Chemoecology 9:119–126.

Baer, B., and P. Schmid-Hempel. 1999. Experimental variation in polyandry affects parasite loads and fitness in a bumble-bee. Nature 397:151–154.

Beekman, M., and B. P. Oldroyd. 2005. Honeybee workers use cues other than egg viability for policing. Biol. Lett. 1:129–132.

———. 2008. When workers disunite: intraspecific parasitism by eusocial bees. Annu. Rev. Entomol. 53:19–37.

Bloch, G., and A. Hefetz. 1999a. Reevaluation of the role of mandibular glands in regulation of reproduction in bumblebee colonies. J. Chem. Ecol. 25:881–896.

———. 1999b. Regulation of reproduction by dominant workers in bumblebee (*Bombus terrestris*) queenright colonies. Behav. Ecol. Sociobiol. 45:125–135.

Bonckaert, W., K. Vuerinckx, J. Billen, R. L. Hammond, L. Keller, and T. Wenseleers. 2008. Worker policing in the German wasp *Vespula germanica*. Behav. Ecol. 19:272–278.

Bonckaert, W., A. Tofilski, F. S. Nascimento, J. Billen, F. L. W. Ratnieks, and T. Wenseleers. 2011. Co-occurrence of three types of egg policing in the Norwegian wasp *Dolichovespula norwegica*. Behav. Ecol. Sociobiol. 65:633–640.

Bourke, A. F. G. 1994. Worker matricide in social bees and wasps. J. Theor. Biol. 167:283–292.

———. 2011. Principles of social evolution. Oxford Univ. Press, Oxford, U.K.

Bourke, A. F. G., and N. R. Franks. 1995. Social evolution in ants. Princeton Univ. Press, Princeton, NJ.

Bourke, A. F. G., and F. L. W. Ratnieks. 2001. Kin-selected conflict in the bumble-bee *Bombus terrestris* (Hymenoptera: Apidae). Proc. R. Soc. Lond. B 268:347–355.

Brown, M. J. F., R. Schmid-Hempel, and P. Schmid-Hempel. 2003. Queen-controlled sex ratios and worker reproduction in the bumble bee *Bombus hypnorum*, as revealed by microsatellites. Mol. Ecol. 12:1599–1605.

Brunner, E., and J. Heinze. 2009. Worker dominance and policing in the ant *Temnothorax unifasciatus*. Insectes Soc. 56:397–404.

Cameron, S. A., H. M. Hines, and P. H. Williams. 2007. A comprehensive phylogeny of the bumble bees (*Bombus*). Biol. J. Linn. Soc. 91:161–188.

Cardinal, S., and B. N. Danforth. 2011. The antiquity and evolutionary history of social behavior in bees. PLoS One 6:e21086.

Crozier, R. H., and P. Pamilo. 1996. Evolution of social insect colonies: sex allocation and kin selection. Oxford Univ. Press, Oxford, U.K.

Duchateau, M. J. 1989. Agonistic behaviors in colonies of the bumblebee *Bombus terrestris*. J. Ethol. 7:141–151.

Duchateau, M. J., and H. H. W. Velthuis. 1988. Development and reproductive strategies in *Bombus terrestris* colonies. Behaviour 107:186–207.

———. 1989. Ovarian development and egg laying in workers of *Bombus terrestris*. Entomol. Exp. Appl. 51:199–213.

Duchateau, M. J., H. H. W. Velthuis, and J. J. Boomsma. 2004. Sex ratio variation in the bumblebee *Bombus terrestris*. Behav. Ecol. 15:71–82.

Endler, A., J. Liebig, T. Schmitt, J. E. Parker, G. R. Jones, P. Schreier, and B. Hölldobler. 2004. Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect. Proc. Natl. Acad. Sci. USA 101:2945–2950.

Endler, A., J. Liebig, and B. Holldobler. 2006. Queen fertility, egg marking and colony size in the ant *Camponotus floridanus*. Behav. Ecol. Sociobiol. 59:490–499.

Estoup, A., M. Solignac, M. Harry, and J.-M. Cornuet. 1993. Characterization of (GT)n and (CT)n microsatellites in two insect species: *Apis mellifera* and *Bombus terrestris*. Nucl. Acids Res. 21:1427–1431.

Foster, K. R., F. L. W. Ratnieks, N. Gyllenstrand, and P. A. Thorén. 2001. Colony kin structure and male production in *Dolichovespula* wasps. Mol. Ecol. 10:1003–1010.

Frank, S. A. 2003. Repression of competition and the evolution of cooperation. Evolution 57:693–705.

Hartmann, A., J. Wantia, J. A. Torres, and J. Heinze. 2003. Worker policing without genetic conflicts in a clonal ant. Proc. Natl. Acad. Sci. USA 100:12836–12840.

Hughes, W. O. H., B. P. Oldroyd, M. Beekman, and F. L. W. Ratnieks. 2008. Ancestral monogamy shows kin selection is key to the evolution of eusociality. Science 320:1213–1216.

Huth-Schwarz, A., A. León, R. Vandame, R. F. A. Moritz, and F. B. Kraus. 2011. Workers dominate male production in the neotropical bumblebee *Bombus wilmattae* (Hymenoptera: Apidae). Front. Zool. 8:13. doi:10.1186/1742-9994-8-13

Kikuta, N., and K. Tsuji. 1999. Queen and worker policing in the monogynous and monandrous ant, *Diacamma* sp. Behav. Ecol. Sociobiol. 46:180–189.

Lopez-Vaamonde, C., J. W. Koning, W. C. Jordan, and A. F. G. Bourke. 2003. No evidence that reproductive bumblebee workers reduce the production of new queens. Anim. Behav. 66:577–584.

Lopez-Vaamonde, C., J. W. Koning, R. M. Brown, W. C. Jordan, and A. F. G. Bourke. 2004. Social parasitism by male-producing reproductive workers in a eusocial insect. Nature 430:557–560.

Lopez-Vaamonde, C., N. E. Raine, J. W. Koning, R. M. Brown, J. J. M. Pereboom, T. C. Ings, O. Ramos-Rodriguez, W. C. Jordan, and A. F. G. Bourke. 2009. Lifetime reproductive success and longevity of queens in an annual social insect. J. Evol. Biol. 22:983–996.

Marshall, T. C., J. Slate, L. E. B. Kruuk, and J. M. Pemberton. 1998. Statistical confidence for likelihood-based paternity inference in natural populations. Mol. Ecol. 7:639–655.

Nanork, P., J. Paar, N. C. Chapman, S. Wongsiri, and B. P. Oldroyd. 2005. Asian honeybees parasitize the future dead. Nature 437:829.

Nanork, P., S. Wongsiri, and B. P. Oldroyd. 2007. Preservation and loss of the honey bee (*Apis*) egg-marking pheromone signal across evolutionary time. Behav. Ecol. Sociobiol. 61:1509–1514.

Ohtsuki, H., and K. Tsuji. 2009. Adaptive reproduction schedule as a cause of worker policing in social Hymenoptera: a dynamic game analysis. Am. Nat. 173:747–758.

Oldroyd, B. P., L. A. Halling, G. Good, W. Wattanachaiyingcharoen, A. B. Barron, P. Nanork, S. Wongsiri, and F. L. W. Ratnieks. 2001. Worker policing and worker reproduction in *Apis cerana*. Behav. Ecol. Sociobiol. 50:371–377.

Paxton, R. J., P. A. Thorén, A. Estoup, and J. Tengö. 2001. Queen-worker conflict over male production and the sex ratio in a facultatively polyandrous bumblebee, *Bombus hypnorum*: the consequences of nest usurpation. Mol. Ecol. 10:2489–2498.

Payne, C. M., T. M. Laverty, and M. A. Lachance. 2003. The frequency of multiple paternity in bumble bee (*Bombus*) colonies based on microsatellite DNA at the B10 locus. Insectes Soc. 50:375–378.

Peeters, C., and J. Liebig. 2009. Fertility signaling as a general mechanism regulating reproductive division of labor in ants. Pp. 220–242 *in* J. Gadau and J. Fewell, eds. Organization of insect societies: from genome to sociocomplexity. Harvard Univ. Press, Cambridge, MA.

Pirk, C. W. W., P. Neumann, and F. L. W. Ratnieks. 2003. Cape honeybees, *Apis mellifera capensis*, police worker-laid eggs despite the absence of relatedness benefits. Behav. Ecol. 14:347–352.

Pirk, C. W. W., P. Neumann, and H. R. Hepburn. 2007. Nestmate recognition for eggs in the honeybee (*Apis mellifera* L.). Behav. Ecol. Sociobiol. 61:1685–1693.

R Development Core Team. 2011. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Ratnieks, F. L. W. 1988. Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. Am. Nat. 132:217–236.

Ratnieks, F. L. W., and P. K. Visscher. 1989. Worker policing in the honeybee. Nature 342:796–797.

Ratnieks, F. L. W., and T. Wenseleers. 2008. Altruism in insect societies and beyond: voluntary or enforced? Trends Ecol. Evol. 23:45–52.

Rousset, F. 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. Mol. Ecol. Res. 8:103–106.

Saigo, T., and K. Tsuchida. 2004. Queen and worker policing in monogynous and monandrous colonies of a primitively eusocial wasp. Proc. R. Soc. Lond. B 271:S509–S512.

Sauter, A., M. J. F. Brown, B. Baer, and P. Schmid-Hempel. 2001. Males of social insects can prevent queens from multiple mating. Proc. R. Soc. Lond. B 268:1449–1454.

Schmid-Hempel, R., and P. Schmid-Hempel. 2000. Female mating frequencies in *Bombus* spp. from Central Europe. Insectes Soc. 47:36–41.

Sramkova, A., C. Schulz, R. Twele, W. Francke, and M. Ayasse. 2008. Fertility signals in the bumblebee *Bombus terrestris* (Hymenoptera: Apidae). Naturwissenschaften 95:515–522.

Starr, C. K. 1984. Sperm competition, kinship, and sociality in the Aculeate Hymenoptera. Pp. 427–464 *in* R. L. Smith, ed. Sperm competition and the evolution of animal mating systems. Academic Press, Orlando, FL.

Stroeymeyt, N., E. Brunner, and J. Heinze. 2007. "Selfish worker policing" controls reproduction in a *Temnothorax* ant. Behav. Ecol. Sociobiol. 61:1449–1457.

Takahashi, J., M. Itoh, I. Shimizu, and M. Ono. 2008. Male parentage and queen mating frequency in the bumblebee *Bombus ignitus* (Hymenoptera: Bombinae). Ecol. Res. 23:937–942.

Takahashi, J., S. J. Martin, M. Ono, and I. Shimizu. 2010. Male production by non-natal workers in the bumblebee, *Bombus deuteronymus* (Hymenoptera: Apidae). J. Ethol. 28:61–66.

Trivers, R. L., and H. Hare. 1976. Haplodiploidy and the evolution of the social insects. Science 191:249–263.

Truett, G. E., P. Heeger, R. L. Mynatt, A. A. Truett, J. A. Walker, and M. L. Warman. 2000. Preparation of PCR-quality mouse genomic DNA with hot sodium hydroxide and tris (HotSHOT). BioTechniques 29:52–54.

Van der Blom, J. 1986. Reproductive dominance within colonies of *Bombus terrestris* (L.). Behaviour 97:37–49.

Van Doorn, A., and J. Heringa. 1986. The ontogeny of a dominance hierarchy in colonies of the bumblebee *Bombus terrestris* (Hymenoptera, Apidae). Insectes Soc. 33:3–25.

Van Honk, C. G. J., P.-F. Röseler, H. H. W. Velthuis, and J. C. Hoogeveen. 1981. Factors influencing the egg laying of workers in a captive *Bombus terrestris* colony. Behav. Ecol. Sociobiol. 9:9–14.

Velthuis, H. H. W., D. De Araujo Alves, V. Imperatriz-Fonseca, and M. J. Duchateau. 2002. Worker bees and the fate of their eggs. Pp. 97–102 *in* J. Bruin, ed. Proceedings of the section experimental and applied entomology of the Netherlands Entomological Society (NEV), Vol. 13. NEV, Amsterdam.

Visscher, P. K. 1989. A quantitative study of worker reproduction in honey bee colonies. Behav. Ecol. Sociobiol. 25:247–254.

Visscher, P. K., and R. Dukas. 1995. Honey bees recognize development of nestmates' ovaries. Anim. Behav. 49:542–544.

Wenseleers, T., and F. L. W. Ratnieks. 2006. Comparative analysis of worker reproduction and policing in eusocial Hymenoptera supports relatedness theory. Am. Nat. 168:E163–E179.

Wenseleers, T., A. Tofilski, and F. L. W. Ratnieks. 2005. Queen and worker policing in the tree wasp *Dolichovespula sylvestris*. Behav. Ecol. Sociobiol. 58:80–86.

Woyciechowski, M., and A. Lomnicki. 1987. Multiple mating of queens and the sterility of workers among eusocial Hymenoptera. J. Theor. Biol. 128:317–327.

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Supporting Information

The following supporting information is available for this article:

Table A1. Details of the study colonies of *Bombus terrestris* and of behavioral sampling (first experiment).
Table A2. Results and sample sizes for microsatellite analysis of male parentage in *Bombus terrestris* (first experiment).
Table A3. Viabilities of queen- and worker-laid haploid eggs in *Bombus terrestris,* with sample sizes for estimation of egg viability and for genetic confirmation of ploidy of eggs (second experiment).

Supporting Information may be found in the online version of this article.

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