

Worker policing in the German wasp *Vespula germanica*

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In some ants, bees, and wasps, workers kill or “police” male eggs laid by other workers in order to maintain the reproductive primacy of the queen. Kin selection theory predicts that multiple mating by the queen is one factor that can selectively favor worker policing. This is because when the queen is mated to multiple males, workers are more closely related to the queen’s sons than to the sons of other workers. Earlier work has suggested that reproductive patterns in the German wasp *Vespula germanica* may contradict this theory, because in some colonies a large fraction of the adult males were inferred to be the workers’ sons, despite the effective queen mating frequency being greater than 2 (2.4). In the present study, we reexamine the *V. germanica* case and show that it does support the theory. First, genetic analysis confirms that the effective queen mating frequency is high, 2.9, resulting in workers being more related to the queen’s sons than to other workers’ sons. Second, behavioral assays show that worker-laid eggs are effectively killed by other workers, despite worker-laid eggs having the same intrinsic viability as queen-laid ones. Finally, we estimate that approximately 58.4% of the male eggs but only 0.44% of the adult males are worker derived in queenright colonies, consistent with worker reproduction being effectively policed. *Key words*: reproductive conflict, Vespinae wasps, *Vespula germanica*, worker policing, worker reproduction. [*Behav Ecol* 19:272–278 (2008)]

Major evolutionary transitions (Maynard Smith and Szathmáry 1995) require the evolution of mechanisms that moderate within-group conflict (Keller 1999; Queller 2000; Ratnieks et al. 2006). One such mechanism is mutual policing, whereby social control is used to either prevent individuals from acting selfishly or reduce the payoffs from doing so (Ratnieks 1988; Frank 1995; Frank 2003; Wenseleers et al. 2004a; Ratnieks and Wenseleers 2005, 2007; Ratnieks et al. 2006; Wenseleers and Ratnieks 2006b). One of the best examples of mutual policing behavior in nature is found in ants, bees, and wasps, where it is used to inhibit workers from producing their own male offspring (Ratnieks 1988; Ratnieks et al. 2006; Wenseleers and Ratnieks 2006a). In most species, workers are unable to mate, but they usually possess functional ovaries and are able to lay unfertilized, male-destined eggs (Trivers and Hare 1976; Bourke 1988; Hammond and Keller 2004; Wenseleers and Ratnieks 2006a). Nevertheless, in most species, egg-laying workers are aggressed or the eggs they lay are eaten by either the mother queen or by other workers, a process referred to as queen policing and worker policing, respectively (reviewed in Ratnieks et al. 2006; Wenseleers and Ratnieks 2006a).

Kin selection theory (Hamilton 1964) predicts that the queen is selected to carry out such policing because she is more related to her sons ($r = 1/2$) than to her daughter workers’ sons (grandsons, $r = 1/4$) (Trivers and Hare 1976). By contrast, the workers are selected to police particularly when the queen mates with multiple males. This is because the workers are then on average more closely related to

queen’s sons (brothers, $r = 0.25$) than to other workers’ sons (a mix of full- and half-nephews, $r < 0.25$) (Starr 1984; Woyciechowski and Lomnicki 1987; Ratnieks 1988). Worker policing, however, can also be selected for under single mating if it has additional benefits, for example, when the policing of reproductive workers increases colony efficiency (Ratnieks 1988) or when the removal of worker-laid eggs, which are all male, could help the workers to cause a female-biased sex allocation ratio (Foster and Ratnieks 2001b).

Evidence supports both relatedness and secondary productivity or sex ratio benefits as important influences on the occurrence of worker policing (reviewed in Hammond and Keller 2004; Wenseleers and Ratnieks 2006a). In particular, worker policing has been found in all species surveyed where queens mate multiple times, for example, in *Apis* honeybees (Ratnieks and Visscher 1989; Halling et al. 2001; Oldroyd et al. 2001), the common wasp *Vespula vulgaris* (Foster and Ratnieks 2001a), and *Pachycondyla* and *Acromyrmex* ants (D’Ettorre et al. 2004; Dijkstra and Boomsma 2004) and also in approximately 20% of the species where queens generally mate only once, for example, in the hornet *Vespa crabro* (Foster et al. 2002) and the ant *Camponotus floridanus* (Endler et al. 2004) (reviewed in Wenseleers and Ratnieks 2006a). This is consistent with limited selection for worker policing in species with low mating frequency due to productivity or sex ratio benefits and with strong, universal selection for worker policing in species with high queen mating frequency due to relatedness effects, possibly in combination with efficiency benefits (Wenseleers and Ratnieks 2006a).

To date, the only possible exception to the pattern that species with high mating frequency generally have low levels of worker reproduction has been found in the yellow jacket *Vespula germanica* (Goodisman et al. 2002). Based on a reanalysis of the original dataset, Hammond and Keller (2004) concluded that an average of 22% of the adult males were the workers’ sons in this species. This was strongly against expectation, because the effective queen mating frequency in this

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species was high, 2.4, and worker reproduction would therefore have been expected to be effectively policed (Goodisman et al. 2002).

Given this highly anomalous result, we here reexamine the *V. germanica* and show that it does support the theory. In particular, we show using behavioral assays that worker-laid eggs are effectively killed by other workers, despite worker-laid eggs having the same intrinsic viability as queen-laid ones. The latter rules out the possibility that policing could be aimed at removing worker-laid eggs of low viability (Gadagkar 2004; Pirk et al. 2004; Nonacs 2006, but see Beekman and Oldroyd 2005; Helanterä et al. 2006). In addition, we estimate that approximately 58.4% of the male eggs but only 0.44% of the adult males are worker derived in queenright colonies. This again implies that worker reproduction is effectively policed, as predicted by theory.

METHODS

Study organism

Our study is based on 10 colonies of the German wasp *V. germanica*, which we found in the vicinity of Leuven, Belgium, in August and September 2005 and 2006 (Table 1). Six of the colonies were nesting underground, and 4 were found under rooftops. Seven colonies could be collected in their entirety and were set up in observation boxes after male and female brood was sampled (see below). The actual nests of the other 3 were inaccessible, and so for those only workers were sampled from the nest entrance (Table 1). All collected colonies were queenright, that is, had a laying mother queen present. Nests have a short annual cycle, being founded in spring by a single overwintered and inseminated queen and dying out at the end of October (Spradbery 1973). All experiments were carried out at the end of September, at a time when colonies naturally produce new queens and males.

Queen mating frequency and worker relatedness

To determine queen mating frequency, we analyzed between 20 and 65 female offspring (queen or worker larvae, pupae, or

adults, mean = 33.0, standard deviation [SD] = 17.7, Table 1) from each of the 10 nests at 2 highly polymorphic microsatellite loci, List2001 and List2003 (Daly et al. 2002). DNA was extracted using the Chelex method, whereby an antenna from an adult or pupa or the head of a larva was frozen in liquid nitrogen and ground up using a plastic pestle, followed by an incubation at 95 °C for 15 min in 200 µL of a 10% Biorad Chelex 100 resin solution. Samples were vortexed and centrifuged before use. Standard polymerase chain reactions (PCRs) were carried out for each locus separately in a 15-µL mixture containing 0.5 µM of the forward and reverse primers, 0.2 mM of each dNTP, 1.5 mM MgCl₂, 1.5 µL of crude DNA extract, 0.6 units of Silverstar *Taq* polymerase (Eurogentec, Seraing, Belgium), and 1× enzyme buffer supplied by the manufacturer. PCR was performed after a touch-down programme, with an initial denaturation for 3 min at 94 °C, followed by 20 cycles consisting of 30 s at 94 °C, 30 s at 56 °C, but decreasing 0.5 °C in each step, and 45 s at 72 °C; 10 cycles consisting of 30 s at 94 °C, 30 s at 46 °C, and 45 s at 72 °C; and a final 10-min extension step at 72 °C. After amplification, 1 µL of the PCR product was mixed with 5.8 µL formamide and 0.2 µL Genescan 500 LIZ size standard (Applied Biosystems, Lennik, Belgium), denatured, and loaded onto an ABI-3130 Avant capillary sequencer. Alleles were called using the supplied Gene Mapper software.

Genotypes of the mother queens were determined by direct genotyping (for the 7 nests that were collected in their entirety and the queens were recovered) or were reconstructed from worker offspring genotypes (for the 3 remaining colonies, Table 1). Once the mother queens' genotypes were established, the Matesoft program (Moilanen et al. 2004) was used to determine the number of males the queens had mated to (paternity) and to assign individual workers to particular patriline. Individual patriline assignments were then used to calculate the effective paternity of each colony (Starr 1984), $M_e = 1 / \sum_i p_i^2$, where p_i is the proportional contribution of the i th male mate. This is the effective number of males the queen mates with, correcting for unequal paternity contribution. The corresponding pedigree worker-worker relatedness was calculated for each colony as $r = 1/4 + 1/2M_e$ (Pamilo 1991). Arithmetic mean relatedness and the

Table 1

Male parentage, queen mating frequency, paternity skew, and sister-sister relatedness for 10 queenright *Vespula germanica* colonies

| Colony | Year collected | No. of diploid brood genotyped | No. of males genotyped | No. of workers' sons detected | No. of fathers detected | Effective queen mating frequency | Paternity skew | Expected paternity skew under equal paternity | Pedigree sister-sister relatedness |
|-----------------|----------------|--------------------------------|------------------------|-------------------------------|-------------------------|----------------------------------|--------------------|---|------------------------------------|
| 17 ^a | 2005 | 65 | 59 | 0 | 4 | 4.00 | 0.00 | 0.05 | 0.38 |
| 18 ^a | 2005 | 22 | 91 | 0 | 2 | 1.98 | 0.02 | 0.06 | 0.50 |
| 20 ^a | 2005 | 57 | 16 | 0 | 4 | 3.96 | 0.01 | 0.05 | 0.38 |
| 23 ^a | 2005 | 22 | 93 | 0 | 3 | 2.78 | 0.11 | 0.09 | 0.43 |
| 35 ^b | 2005 | 25 | 0 | 0 | 4 | 3.05 | 0.32 | 0.11 | 0.41 |
| 1 ^a | 2006 | 52 | 21 | 1 | 3 | 2.97 | 0.01 | 0.04 | 0.42 |
| 5 ^b | 2006 | 20 | 0 | 0 | 4 | 3.39 | 0.20 | 0.14 | 0.40 |
| 7 ^b | 2006 | 20 | 0 | 0 | 4 | 3.92 | 0.03 | 0.14 | 0.38 |
| 8 ^a | 2006 | 27 | 43 | 0 | 4 | 2.31 | 0.56 | 0.10 | 0.47 |
| 12 ^a | 2006 | 20 | 49 | 0 | 3 | 2.41 | 0.30 | 0.10 | 0.46 |
| Mean | | $\bar{x} = 33^d$ | $\bar{x} = 53^c$ | $\bar{x} = 0.27\%^d$ | $\bar{x} = 3.5^d$ | $\bar{x}_q = 2.92^c$ | $\bar{x} = 0.16^d$ | $\bar{x} = 0.09^d$ | $\bar{x} = 0.42^d$ |
| [95% CL] | | | | [0.07%, 0.99%] | [3.0, 4.0] | [2.47, 3.56] | [0.02, 0.29] | [0.06, 0.11] | [0.39, 0.45] |

^a Mother queen genotyped.

^b Maternal genotypes reconstructed from the worker genotypes.

^c Mean for the 7 colonies from which male brood could be sampled.

^d Arithmetic mean.

^e Harmonic mean.

corresponding harmonic mean paternity were calculated as population summary values. Harmonic mean is used because relatedness and paternity are inversely related to each other (Boomsma and Ratnieks 1996).

Two types of errors potentially confound effective paternity estimates: nondetection and nonsampling (Boomsma and Ratnieks 1996). The nondetection error is the probability that father males have identical multilocus genotypes and hence cannot be distinguished; nonsampling error is the probability that one or more patriline are actually present but are not sampled (Foster and Ratnieks 2001a; Nielsen et al. 2003). The magnitude of both errors was evaluated using the equations in Foster and Ratnieks (2001a). The inequality in paternity contributions across father males in all colonies was evaluated using the paternity skew index $S = (M_t - M_e)/(M_t - 1)$ (Pamilo and Crozier 1996), where M_t is the total number of male mates and M_e the effective number of mates, for the given worker array. This index rises from 0 to 1 as paternity becomes more unequal. As a result of sampling error, unequal paternity is expected even when the fathers have equal total numbers of offspring (Foster and Ratnieks 2001a). Therefore, the paternity skews for each colony were compared with those expected under equal paternity using a paired *t*-test. Based on simulated binomial or multinomial distributions, we calculated that equal paternity should result in skews of 0.05–0.14 with the observed number of male mates and female brood analyzed (Table 1).

Behavioral observations of worker reproduction and policing

To carry out policing bioassays, 7 *V. germanica* colonies were transferred to 15 (width) × 17 (depth) × 40 cm (height) wooden observation boxes maintained outside the Institute for Botany and Microbiology in Leuven. Colonies were allowed to fly freely and forage naturally. Of the 7 nests, 4 successfully reestablished (colonies 17, 18, 20, and 23) and were used to carry out policing assays following a slightly modified version of the protocol of Foster et al. (2002). Each trial involved 3 colonies: a queenright discriminator colony, a queenright colony that acted as a source of queen-laid eggs, and a queenless colony that acted as a source of worker-laid eggs (Table 2). Because the colonies used were small (ca. 200 workers), only 1.6% of the workers had active ovaries, and reproductive workers can only lay around 1 egg per day (see Results), we assumed that most or all of the eggs laid in our queenright source colonies were queen laid. The combs used

from the source colonies were emptied a day before the trial so that the eggs used in the trials were approximately 1 day old. The eggs were then collected on a glass plate using flexible forceps, so we could check that the collected eggs were intact. Only the undamaged eggs were used in the trials, damaged eggs were discarded. Subsequently, the queen-laid and worker-laid eggs were glued into an empty test comb using 1% methylcellulose glue in alternating rows of queen-laid and worker-laid eggs. The test comb was the lowest comb of each discriminator colony and consisted of large cells which are used to rear both males and queens; the comb itself was emptied shortly before each experiment and glued onto a thin iron wire attached to a piece of children's modeling clay, allowing the comb to be taken in and out of the colony, thereby enabling us to monitor egg survival. The numbers of queen-laid and worker-laid eggs remaining were checked after 1 h, 1 day, and 2 days. Policing behavior was recorded using a small infrared night vision camera (Conrad Electronic, Oldenzaal, The Netherlands, catalog number 190865) placed at the bottom of each nest box and connected to a computer using an Avermedia EZMaker framegrabber and VirtualDub (<http://www.virtualdub.org/>) software. This allowed time-lapse digital video recording (5 frames/s) onto the computer hard disk. We did 4 trials with a total of 114 queen-laid eggs and 114 worker-laid eggs (Table 2). Observations were carried out between the 15th and 18th of September 2005.

The statistical significance of differences in the policing rate of introduced worker-laid and queen-laid eggs was tested both at the level of each individual trial using Fisher's Exact tests and across all trials using a generalized linear model (GLM) with binomial error structure and logit link function, in which QUEEN VS. WORKER-LAID and TRIAL were included as factors in the analysis. Because we were limited by the number of healthy colonies we had available, some colonies were used twice in our assays, either as a source of eggs or as a discriminator colony (Table 2). This means that the 4 trials we performed cannot be considered as fully independent.

Egg viability

To exclude the possibility that worker-laid eggs could have a lower intrinsic viability than queen-laid ones (Pirk et al. 2004; Nonacs et al. 2006), we transferred 50 queen-laid eggs and 138 worker-laid eggs into combs, which we incubated at 25 °C and 68% relative humidity (Table 3). We also incubated combs with nontransferred eggs in their original combs under

Table 2
Proportions of worker-laid and queen-laid eggs policed after 1 h, 1 day, and 2 days in 2 queenright colonies

| Trial | Source colony of eggs | | Discriminator colony | No. of eggs surviving after | | | | | Statistical significance of difference in survival after 2 days |
|-----------|-----------------------|-----------------|----------------------|-----------------------------|---------------------|---------|---------|---------|---|
| | Queen-laid | Worker-laid | | Egg type | Initial no. of eggs | 1 h | 1 day | 2 days | |
| 1 | 23 | 18 ^a | 17 | Queen-laid | 23 | 11 | 8 | 8 | <i>P</i> = 0.004 |
| | | | | Worker-laid | 23 | 1 | 0 | 0 | |
| 2 | 17 | 20 ^a | 23 | Queen-laid | 23 | 23 | 15 | 7 | <i>P</i> = 0.01 |
| | | | | Worker-laid | 23 | 20 | 4 | 0 | |
| 3 | 17 | 17 ^a | 17 | Queen-laid | 36 | 22 | 16 | 14 | <i>P</i> = 0.0002 |
| | | | | Worker-laid | 36 | 12 | 1 | 0 | |
| 4 | 23 | 23 ^a | 23 | Queen-laid | 32 | 28 | 21 | 7 | <i>P</i> = 0.01 |
| | | | | Worker-laid | 32 | 23 | 4 | 0 | |
| Total (%) | | | | Queen-laid | 114 | 84 (74) | 60 (53) | 36 (32) | |
| | | | | Worker-laid | 114 | 56 (49) | 9 (8) | 0 (0) | |

The statistical significance of the differences in survival of queen-laid and worker-laid eggs is calculated using 2-sided Fisher's Exact tests.

^a Queenless fragment of the original queenright colony.

Table 3
Survival and hatching of transferred and nontransferred queen-laid and worker-laid eggs

| Egg type | Source colony | Initial no. of eggs | No. of eggs surviving or hatched after | | | | | | Percent hatched | Statistical significance of difference in hatching rate | |
|---|-----------------|---------------------|--|--------|--------|--------|--------|--------|-----------------|---|------------|
| | | | 1 day | 2 days | 3 days | 4 days | 5 days | 6 days | | | |
| a) Transferred <i>Vespa germanica</i> queen-laid and worker-laid eggs | | | | | | | | | | | |
| Queen-laid | 17 | 25 | 9 | 9 | 7 | 6 | 6 | 5 | 20 | $P = 0.19$ | |
| | 23 | 25 | 16 | 14 | 11 | 7 | 7 | 7 | 28 | | |
| Worker-laid | 17 ^a | 25 | 10 | 5 | 3 | 3 | 3 | 3 | 12 | | |
| | 18 ^a | 66 | 45 | 42 | 37 | 31 | 31 | 30 | 45 | | |
| | 20 ^a | 10 | 8 | 1 | 1 | 1 | 1 | 1 | 10 | | |
| | 23 ^a | 37 | 26 | 25 | 17 | 12 | 9 | 9 | 24 | | |
| Total | Queen-laid | 50 | 25 | 23 | 18 | 13 | 13 | 12 | 24 | | |
| | Worker-laid | 138 | 89 | 73 | 58 | 47 | 44 | 43 | 31 | | |
| b) Nontransferred <i>V. germanica</i> queen-laid and worker-laid eggs | | | | | | | | | | | |
| Queen-laid | 17 | 48 | 47 | 39 | 39 | 38 | 38 | 38 | 79 | | $P = 0.15$ |
| | 23 | 55 | 51 | 51 | 51 | 51 | 49 | 49 | 89 | | |
| Worker-laid | 17 ^a | 78 | 68 | 67 | 62 | 59 | 59 | 59 | 76 | | |
| | 18 ^a | 293 | 276 | 273 | 244 | 232 | 231 | 231 | 79 | | |
| | 20 ^a | 124 | 122 | 118 | 118 | 115 | 112 | 112 | 90 | | |
| | 23 ^a | 297 | 292 | 279 | 258 | 250 | 250 | 250 | 84 | | |
| Total | Queen-laid | 103 | 98 | 90 | 90 | 89 | 87 | 87 | 84 | | |
| | Worker-laid | 792 | 758 | 737 | 682 | 656 | 652 | 652 | 82 | | |

Statistical significances of the differences in hatching rate are calculated using 2-sided Fisher's Exact tests.

^a Queenless fragment of the original queenright colony.

the same conditions (Table 3). All eggs were approximately 1 day old. Every 24 h, the combs were inspected to count the number of surviving eggs and the number of larvae that had hatched. Hatched larvae were removed, so they would not be counted twice. The combs were monitored for up to 6 days because the development of an egg to a first instar larva takes approximately 5 days (Spradbery 1971).

Percentage of worker-laid eggs and parentage of adult males

Following the method of Helanterä et al. (2006), we estimated the percentage of worker-laid eggs in natural queenright colonies from the queen and worker fecundities as $A = (n_W \cdot p \cdot e_W) / (n_W \cdot p \cdot e_W + e_Q)$, where n_W is the mean colony size, p the proportion of workers with activated ovaries, and e_W and e_Q the number of eggs laid by a single worker and by the queen per day. The mean colony size n_W of reproductive stage queenright colonies in August/September is 1331 workers ($n = 26$ colonies, Spradbery 1971). The number of eggs laid per day by the queen e_Q at the same time is 154 ($n = 26$ colonies, Spradbery 1971). To establish the proportion of workers that had activated ovaries (p) we dissected a total of 1008 workers taken from 5 mature queenright nests. Assuming that the egg-laying rates of workers with active ovaries in queenright and queenless colonies are identical, we determined e_W by allowing 11–41 workers from 3 queenless colonies each to lay eggs for 5 days in an empty comb and dissecting them at the end. Ovary active workers were defined as those having ovaries containing oocytes at least half the size of a freshly laid egg (Foster and Ratnieks 2001a). From A , we calculated the proportion of male eggs that were worker laid as $B = A / (A + [1 - A] \cdot m)$, where m is the proportion of the queen-laid eggs that are male, which has been estimated at 13.0% ($n = 26$ colonies, Spradbery 1971). Ninety-five percent confidence limits (CLs) on A and B were calculated using a Monte Carlo procedure as described in Helanterä et al. (2006).

To determine the proportion of adult males that were the queen's and the workers' sons, a total of 372 (N) males from

7 queenright colonies (mean = 53, SD = 30.5, Table 1) were genotyped using the same 2 loci used to determine queen mating frequency. Loci are informative only if the paternal and maternal alleles of the workers differ, that is, if a worker's son can be distinguished from a queen's sons when he inherits a paternal allele not present in the queen (Foster et al. 2000). The proportion of worker-produced males for each nest was estimated as $N_w / (P_j N_j)$ where N_j and N_w are the total number of males analyzed for the j th nest and the number of detected worker's sons, respectively, and P_j is the power of detecting a worker's son in the j th nest (Foster and Ratnieks 2001a). The power for detecting workers' sons was calculated following Foster and Ratnieks (2001a).

RESULTS

Allelic diversity, worker relatedness, and queen mating frequency

Genetic variation at the 2 microsatellite loci studied was very high, with 16 and 11 detected alleles and expected heterozygosities of 0.86 and 0.83 at loci LIST2001 and LIST2003, respectively. The genotypes of the analyzed diploid brood show that all mother queens had mated to multiple males and that most (6/10) had mated with 4 males; the mean number of male mates was 3.5. The harmonic mean effective paternity was only slightly lower, 2.92 (Table 1). The mean paternity skew was 0.16, which was not significantly greater than the expected value (0.09) if all fathers contributed equally (paired t -test, $t = 1.27$, $P = 0.24$, Table 1). The observed paternities translate into a mean sister–sister pedigree relatedness of 0.42, which is significantly lower than 0.5 (single-sample t -test, $t = -4.72$, 1-sided $P = 0.0005$, Table 1). Hence, workers are significantly more related to the queen's sons ($r = 0.25$) than to other workers' sons ($r = 0.42/2 = 0.21$). Given the observed heterozygosities, the nondetection error was low, 0.02 (cf. Foster and Ratnieks 2001a), and with an average of 33 diploid brood per colony being genotyped

(Table 1), the mean nonsampling probabilities of males with $P = 50\%$, 33% , or 25% paternities were only $(1 - P)^{33} = 1.2 \times 10^{-10}$, 1.5×10^{-6} , and 7.5×10^{-5} , respectively, on average. Hence, neither of these errors likely affected our paternity estimates.

Behavioral observations of worker reproduction and policing

Summing across all 4 trials, after 1 h more than half of the introduced worker-laid eggs were policed, whereas 74% of the queen-laid eggs still remained (Table 2). Furthermore, 60 of 114 (53%) queen-laid eggs but only 9 of 114 (8%) worker-laid eggs survived for 1 day (Table 2). After 2 days, none of the worker-laid eggs remained whereas 36 of 114 (32%) of the queen-laid eggs remained (Table 2). These differences in the policing rate of worker-laid and queen-laid eggs after 2 days were highly significant, both when tested for each trial individually (Fisher's Exact test, P between 0.0002 and 0.01, Table 2) and when trial was included as a factor (GLZ, factor QUEEN VS. WORKER-LAID: $P = 4 \times 10^{-14}$). In the latter analysis, the first-order interaction effect TRIAL \times QUEEN VERSUS WORKER-LAID was not significant, $P = 1$, meaning that differences in policing rates between trials were not significant. The low survival of queen-laid eggs after 2 days (32%) was most likely caused by the procedure of transferring eggs because in the viability experiments only 18 out of 50 (36%) of the transferred queen-laid eggs survived for 2 days (Table 3a). The policing of worker-laid eggs occurred irrespective of whether the source and discriminator colonies were originating from the same colonies or whether they came from entirely different colonies (Table 2). This demonstrates that the chemical signal used to differentiate between worker-laid and queen-laid eggs is probably universal, rather than colony specific. Finally, video recordings clearly revealed that it was not the queen but the workers that policed worker-laid eggs. In fact, in 3 of the 4 trials the queen did not even visit the test comb during the first day that the comb was introduced.

Egg viability

There was no difference in the overall hatch rate of transferred or untransferred queen-laid and worker-laid eggs, either when tested on all data pooled together (Fisher's Exact test, $P = 0.19$ and $P = 0.15$, respectively) or when tested in a pairwise fashion for the 2 colonies, 17 and 23, for which we had both queenright and queenless colony fragments available (paired t -test, $t = 3.0$, $P = 0.20$ and $t = 4.0$, $P = 0.16$, respectively) (Table 3). Hence, worker-laid eggs did not have lower viability than queen-laid ones. The overall survival of transferred eggs, however, was lower than that of nontransferred eggs (queen-laid eggs: Fisher's Exact test, $P = 6 \times 10^{-13}$; paired t -test, $t = 60.0$, $P = 0.01$; worker-laid eggs: Fisher's Exact test, $P = 5 \times 10^{-29}$; paired t -test, $t = 31.00$, $P = 0.021$; Table 3).

Percentage of worker-laid eggs and male parentage

Of 1008 examined workers from 5 colonies (mean = 201.6, SD = 84.0), 16 (1.6%) had activated ovaries of which 3 (0.30%) contained nearly mature oocytes (>90% full-size). These estimates are close to the percentage of workers with active ovaries in queenright colonies of a North American population of *V. germanica*, 0.6% (Ross 1985). Given a mean colony size in August/September of 1331 workers (Spradbery 1971), colonies therefore contain an average of 21 egg-laying workers. In 3 queenless colonies, a total of 27 reproductive workers (32% of the dissected workers) laid 178 eggs over

a period of 5 days. Hence, the mean number of eggs laid per reproductive worker per day (e_w) was 1.32. This leads to an estimated 15.4% (A) and 58.4% (B) of all eggs and male eggs, respectively, being worker-laid in queenright colonies (95% CL on A and B: [8.2%, 25.1%] and [40.7%, 72.2%]).

In contrast to these high estimates of the percentage of eggs that were worker laid, the genotyping results show that only 0.27% (1/372, 95% CL: [0.07%, 0.99%]) of the screened adult males were the workers' sons (Table 1). The average power for detecting workers' sons (Foster and Ratnieks 2001a) was 61%. Taking this into account results in an estimated 0.44% of the adult males being workers' sons (95% CL: [0.11%, 1.61%]). The effective number of assignable males was $P \times N = 226.7$. The clear discrepancy between the high percentage of male eggs that were estimated to be worker laid (58.4%) and the low percentage of adult males that were workers' sons (0.44%) is consistent with the forementioned observations which show that worker-laid eggs are effectively policed.

DISCUSSION

Our results demonstrate that Belgian populations of *V. germanica* are characterized by a high effective queen mating frequency, 2.9 (Table 1)—an estimate that is close to that obtained for an Australian population of this species, 2.4 (Goodisman et al. 2002). The estimate is also in the range of effective queen mating frequencies observed in other large-colony *Vespula*, where the effective paternity is either not significantly different from 2 (*V. vulgaris*, Foster and Ratnieks 2001a) or significantly higher (3.3 in *Vespula squamosa*, Ross 1986, and 5.2 in *Vespula maculifrons*, Goodisman et al. 2007). The high effective paternity and approximately equal sperm use of the male mates form a nice contrast, however, with results obtained for other vespines such as the hornet *Vespa crabro*, *Dolichovespula*, and the small-colony species *Vespula rufa*, where paternity skews are high and effective queen mating frequencies significantly below 2 (1.1, 1–1.4, and 1.5, respectively, Foster et al. 1999, 2001; Wenseleers et al. 2005).

Given this high effective paternity, worker–worker relatedness was relatively low, 0.42 and workers were genetically more related to the sons of the queen (brothers, $r = 0.25$) than to the sons of other workers (full- and half-nephews, $r = 0.21$). On this basis, it was predicted that workers should police each others' reproduction (Ratnieks 1988). Our results confirm this prediction and demonstrate that workers selectively kill worker-laid eggs to favor those laid by the queen (Table 2). In addition, we estimated that 58.4% of the male eggs but only 0.44% of the adult males were the workers' sons, again consistent with worker reproduction being effectively policed. Finally, we demonstrated that the lower survival of workers' sons compared with the queen's sons was not due to a lower intrinsic viability of worker-laid eggs, against the hypothesis of Pirk et al. (2004) to explain worker policing in the honeybee. Recently, absence of large viability differences between queen-laid and worker-laid eggs have also been shown in *V. vulgaris* (Helanterä et al. 2006), and experiments have also refuted the hypothesis for the honeybee itself (Beekman and Oldroyd 2005). That we found only a very small proportion of the adult males to be the workers' sons means that the anomalous genotypes found in one colony by Goodisman et al. (2002) were most likely caused by queen takeover, which is common in vespine wasps (Foster and Ratnieks 2001c), and not by worker reproduction. Indeed, this was pointed out as a possibility by Goodisman et al. (2002).

Altogether, our results are important in that they provide an important data point to strengthen the relatedness hypothesis for the evolution of worker policing (Ratnieks 1988;

Wenseleers and Ratnieks 2006a). Indeed, comparison with the 2 closely related species *V. vulgaris* and *V. rufa* shows the power of the hypothesis in predicting reproductive patterns. As in *V. germanica*, in *V. vulgaris* paternity is high and workers effectively police worker reproduction so that a high proportion of the male eggs but not the adult males are the workers' sons (Foster and Ratnieks 2001a; Helanterä et al. 2006). By contrast, in *V. rufa*, effective paternity is significantly below 2, a significant percentage (11%) of the adult males are the workers' sons, and worker-laid eggs are policed by the queen but not by the workers (Wenseleers et al. 2005). In addition, policing is less effective in *V. rufa* than in *V. vulgaris* and *V. germanica*, as expected from the fact that it is more difficult for a single queen to control the whole colony than it is for the workers to control each other (Ratnieks 1988; Ratnieks and Reeve 1992). A lower policing effectiveness in *V. rufa* probably also explains why a much higher percentage of the workers attempt to reproduce in that species, 9.2%, as opposed to only 1.6% and 1.0% in *V. germanica* and *V. vulgaris*, respectively (Wenseleers et al. 2004a; Wenseleers et al. 2004b; Wenseleers and Ratnieks 2006b). That is, there is more to gain from laying eggs if relatively more of the workers' eggs escape policing (Wenseleers et al. 2004a; Wenseleers et al. 2004b).

On the other hand, against the relatedness hypothesis for the evolution of worker policing, it remains true that worker policing has also been observed in species with low queen mating frequency, including the hornet *Vespa crabro* (Foster et al. 2002) and some ants (Wenseleers and Ratnieks 2006a). This demonstrates that worker policing can also be favored if it has other benefits, for example, by increasing colony productivity (Ratnieks 1988) or causing a more female-biased sex ratio (the workers' optimum, Foster and Ratnieks 2001b) (Hammond and Keller 2004; Wenseleers and Ratnieks 2006a). Productivity benefits are expected particularly when policing prevents workers from laying eggs via aggression; by contrast, policing via egg eating is more likely to result in sex ratio benefits, as egg eating alone is unlikely to make the colony more productive. Nevertheless, our results are important in that they show that *V. germanica* forms no exception to the rule that worker reproduction should be effectively policed in a species where queens mate multiple times (Ratnieks 1988). Indeed, any exception to this pattern would be a much bigger challenge to the theory than the occurrence of worker policing in species with single mating, which can be readily explained (Ratnieks 1988; Foster and Ratnieks 2001b).

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