Colony stage and not facultative policing explains pattern of worker reproduction in the Saxon wasp

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Abstract

Inclusive fitness theory predicts that in colonies of social Hymenoptera headed by a multiple-mated queen, workers should benefit from policing eggs laid by other workers. Foster & Ratnieks provided evidence that in the vespine wasp *Dolichovespula saxonica*, workers police other workers' eggs only in colonies headed by a multiple-mated queen, but not in those headed by a single-mated one. This conclusion, however, was based on a relatively small sample size, and the original study did not control for possible confounding variables such as the seasonal colony progression of the nests. Our aim, therefore, was to reinvestigate whether or not facultative worker policing occurs in *D. saxonica*. Remarkably, our data show that in the studied Danish population, there was no correlation between worker–worker relatedness and the percentage of worker-derived males. In addition, we show that variability in cuticular hydrocarbon profiles among the workers did not significantly correlate with relatedness and that workers therefore probably did not have sufficient information on queen mating frequency from the workers' cuticular hydrocarbon profiles. Hence, there was no evidence that workers facultatively policed other workers' eggs in response to queen mating frequency. Nevertheless, our data do show that the seasonal progression of the nest and the location in which the males were reared both explain the patterns of worker reproduction found. Overall, our results suggest that the earlier evidence for facultative worker policing in *D. saxonica* may have been caused by accidental correlations with certain confounding variables, or, alternatively, that there are large interpopulation differences in the expression of worker policing.

Keywords: *Dolichovespula saxonica*, facultative worker policing, inclusive fitness theory, reproductive conflict, social insects, Vespinae wasps

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Introduction

Social insects are well known for their cooperation, but the nonclonal structure of their colonies also leads to genetic conflicts among society members (Ratnieks & Reeve 1992; Bourke & Franks 1995; Queller & Strassmann 1998; Queller 2000; Bourke 2005; Ratnieks *et al.* 2006). One of these conflicts is the conflict between the

mother queen and her worker offspring over the production of males (Trivers & Hare 1976; Bourke 1988; Hammond & Keller 2004; Ratnieks *et al.* 2006; Wenseleers & Ratnieks 2006a). Conflict over male parentage arises owing to the fact that in most species, the workers, though unable to mate, retain the capacity to lay unfertilized, male eggs (Bourke 1988) and that both the mother queen and the workers are each most related to their own sons. An important mechanism, however, that can resolve this conflict is worker policing, the phenomenon whereby workers prevent other workers from

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successfully reproducing in order to help maintain the reproductive primacy of the queen (reviewed in Ratnieks & Wenseleers 2008). Inclusive fitness theory (Hamilton 1964) predicts that worker policing should be selected for particularly in species with multiple-mated queens, because workers are then on average more closely related to the queen's sons than to the sons of other workers (Starr 1984; Ratnieks 1988). In line with this prediction, worker policing was first discovered in the Western honeybee (Ratnieks & Visscher 1989), a species where queens indeed mate with multiple males (reviewed in Tarpy *et al.* 2004).

Since this initial discovery, worker policing has also been found to occur in Asian honeybees, several vespine wasps and several ant species (reviewed in Wenseleers & Ratnieks 2006a). Furthermore, the predicted role of relatedness in selecting for worker policing has found support in a large meta-analysis of over 100 species of ants, bees and wasps, which showed that a reduction in worker–worker relatedness across the phylogeny of the species studied was correlated with an increased incidence of worker policing and a reduced percentage of adult worker-derived males (Wenseleers & Ratnieks 2006a). Nevertheless, the approach of this meta-analysis can be criticized because phylogenetically related groups with varying queen mating frequency might also differ in many other characteristics, which could confound the comparison. To resolve this issue, several studies have tried to relate variation in male parentage to intraspecific variation in queen mating frequency in species where queens can be either single or multiple mated (reviewed in Wenseleers & Ratnieks 2006a). The argument is that if workers have sufficient information regarding the queen mating frequency in their colony, they should police other workers' eggs in colonies headed by a multiple-mated queen, but not in those headed by a single-mated one. Such behaviour is known as 'facultative worker policing' and so far has only found support in a single study on the vespine wasp *Dolichovespula saxonica*, where genetic data indeed suggested that workers only eat worker-laid eggs in colonies headed by a multiple-mated queen (Foster & Ratnieks 2000). Nevertheless, this conclusion has met with some scepticism, given that it was based on a relatively small sample size, and that the original study did not control for possible confounding variables, such as the stage of colony development at which the colonies were sampled, or the location inside the nest where the males were reared. The stage of colony development is expected to be important, given that both empirical data and recent models have shown that both worker reproduction and policing are likely to depend on the stage of colony growth (Duchateau & Velthuis 1988; Bloch & Hefetz 1999; Wenseleers *et al.* 2004; Ohtsuki & Tsuji

2009; Moore & Liebig 2010). This is because worker reproduction at an early stage would greatly hinder the growth of the work force and therefore come at a cost to the workers' future inclusive fitness, leading to worker policing and/or self-restraint. The location in which the males were reared, in turn, is expected to be important given that the queen might not be able to patrol and police worker-laid eggs as efficiently in all areas in the colony and that in vespine wasps, males reared in different localities in the nest are subject to different levels of kin competition (e.g. competing with the rearing of new sisters queens when reared in the large-celled combs).

Given that facultative worker policing is considered to be an extremely strong test case to assess the role of relatedness in selecting for worker policing, the aim of our study was to re-examine facultative worker policing in *D. saxonica*, using a more extensive set of data from a Danish population. A reassessment of whether or not facultative policing occurs in *D. saxonica* was desirable, given that a robust test of the relationship between levels of policing and relatedness requires (i) adequate control of possible confounding variables, such as the stage of colony development (Wenseleers *et al.* 2004; Ohtsuki & Tsuji 2009; Moore & Liebig 2010), (ii) verification that workers have sufficient information available to detect the variation in colony kin structure, presumably through assessment of variation in cuticular hydrocarbon profiles and (iii) the ability of the workers to facultative adjust their behaviour in response to variation in queen mating frequency. Hence, in contrast to the original study, we made detailed measurements of and controlled for various possible confounding variables, including the stage of colony development at which the colonies were sampled and the location in the nest where male pupae were reared. In addition, we investigated whether the workers' cuticular hydrocarbon profiles contained sufficient information on queen mating frequency to allow the workers to facultatively adjust their policing behaviour in response to queen mating frequency. Cuticular hydrocarbons have been known to play an important role in chemical communication in insects societies (e.g. reviewed in Howard & Blomquist 2005; Blomquist & Bagnères 2010) but only limited information exists about their possible use in the assessment of the colony-specific relatedness structure (Boomsma *et al.* 2003; Dani *et al.* 2004). It is clear, however, that workers require such information to be able to facultatively police worker-laid eggs in response to queen mating frequency. In the context of our study, we hypothesized that multiple mating should lead to a higher genetic variability and a higher chemical variability in the cuticular hydrocarbon profiles of workers. Lastly, to further test for facultative

adjustment of the workers' behaviour in response to queen mating frequency, we also collected detailed data on the investment sex ratio and tested whether workers facultatively manipulate the colony sex ratio depending on the colony relatedness structure (cf. Boomsma & Grafen 1990; Sundström 1994; Sundström *et al.* 1996; Meunier *et al.* 2008; West 2009).

Material and methods

Study species

The Saxon wasp, *Dolichovespula saxonica*, is a monogynous, eusocial yellowjacket with an annual life cycle (Greene 1991). Queens emerge from hibernation in spring and then search for suitable nesting sites. Nests are typically found in shrubs or trees, under eaves of buildings, under porches or in wall cavities (Archer 2006). Once an appropriate nesting site has been found, the queen produces a first generation of workers, which then encloses and takes over foraging, nest construction and brood provisioning. Mature colonies typically consist of a single queen, up to a few hundred workers, one small-celled comb from which one to three generations of workers are reared (as well as males at the end of the season), plus one to four combs consisting of large cells from which a single generation of sexuals are reared (gynes and males) (Greene 1991; Archer 2006). Production of sexuals begins relatively early in colony life, overlaps substantially with the rearing of workers and occupies an increasing proportion of colony effort as the season progresses (Greene 1984, 1991). At the end of the season, most new queens and males eventually leave the nest to mate, after which the queens enter protected locations to overwinter, the other colony members die, and the nest is abandoned (Greene 1991).

Colony sampling and measurement of key parameters

In the summer of 2006 and 2009, 23 colonies of *D. saxonica* were collected on the island of Zealand, Denmark, and in 11 of these, a healthy mother queen was found to be present (Table 1). The remainder was queenless, perhaps due to the occurrence of worker matricide, which is known to be common in *Dolichovespula* (Foster & Ratnieks 2001b; Foster *et al.* 2001; Wenseleers & Ratnieks 2006a). *D. saxonica* has aerial, well-screened nesting sites, e.g. under eaves of buildings or under porches (Schoeters & Wenseleers 2005; personal observations), which are easily accessible and collectable. Therefore, it is very unlikely that if a queen was present, she was lost during collection of the colonies. For each colony, the total number of adult workers as well as the surface areas of all combs was measured. Given

that both empirical data and recent models have shown that both worker reproduction and worker policing are likely to depend on the stage of colony growth (Duchateau & Velthuis 1988; Bloch & Hefetz 1999; Wenseleers *et al.* 2004; Ohtsuki & Tsuji 2009; Moore & Liebig 2010), we calculated a single parameter that would be representative of the stage of colony development. This parameter S was calculated as the total comb area relative to the predicted total comb area if the nest would be fully completed. The latter figure could be calculated given that the small-sized top comb was fully completed in all colonies and that the surface ratio between a given comb and the comb built below it stays constant throughout the season (we inferred it to be 1.3 ± 0.2 SD, 1.0 ± 0.1 SD and 0.8 for the different successive layers of combs). Consequently, the parameter S was bound between 0 for a newly found nest and 1, for a fully completed one. In addition, we estimated the numerical sex ratios (expressed as the proportion of female sexuals produced, i.e. the number of gynes divided by the total number of gynes and males present) by counting the number of male and gyne pupae present in the combs. These numerical sex ratios were transformed to investment sex ratios (expressed as female investment) based on dry weight measurements of 20 gyne pupae, 20 male pupae collected from small-celled combs and 41 male pupae collected from large-celled combs, from a total of nine different colonies. Investment ratios measure the sex ratio whilst accounting for differences in the energy required to rear members of the two sexes (Trivers & Hare 1976; Bourke 2005) and were calculated in the same way as the numerical sex ratio, except that the number of gynes and males produced were each weighted by their respective dry weights and that males reared in large-celled combs and small-celled combs were considered separately. Dry weight measurements were obtained by drying the pupae for 72 h at 56 °C (Dijkstra & Boomsma 2008).

Microsatellite genotyping

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, ground up and incubated at 95 °C for 15 min in 200 µL of a 10% Biorad Chelex 100 resin solution. Three microsatellite loci developed for *Vespula rufa* were used: *Rufa05*, *Rufa13* and *Rufa18* (Thorén *et al.* 1995). Polymerase chain reactions (PCRs) were performed in 10-µL multiplex reactions using the Qiagen multiplex kit and with reactions containing 1 µL of template DNA, 5 µL of master mix, 0.2 µL of a 10 µM solution of each primer and 2.8 µL of Rnase-free H_2O. Primers were fluorescently labelled

Table 1 Data on 23 collected *Dolichovespula saxonica* colonies, including colony number, collection date, presence of the queen (QR, queenright; QL, queenless), the stage of colony development S, colony size (number of adult workers), percentage of reproductive workers (defined as having ovaries containing an oocyte at least half the size of a freshly worker-laid egg), number of workers, worker pupae and males genotyped, the absolute and effective queen mating frequency and pedigree sister–sister relatedness

Colony	Status	Collection date	S	Colony size	% of reproductive workers	No. of workers and worker pupae genotyped	Queen mating frequency	Effective queen mating frequency	Pedigree sister–sister relatedness	No. of males genotyped Pupae from large cells Total	Worker produced (%)	Pupae from small cells Total	Worker produced (%)
1	QR	9 July 2006	0.73	216	3.7	38	3	2.77	0.43	20	0.0	34	98.7
3	QR	12 July 2006	0.64	96	3.1	71	1	1.00	0.75	25	0.0	3	0.0
7	QR	14 July 2006	0.88	208	3.8	40	2	1.60	0.56	20	0.0	20	84.2
16	QR	11 July 2009	0.78	141	5.0	22	3	2.85	0.43	20	12.1	20	72.3
17	QR	11 July 2009	0.71	87	5.0	21	2	2.00	0.50	21	11.8	20	49.4
22	QR	14 July 2009	0.87	65	21.5	32	2	1.54	0.58	20	45.7	20	51.4
23	QR	14 July 2009	0.89	131	12.5	30	1	1.00	0.75	20	0.0	20	100.0
25	QR	15 July 2009	0.71	112	5.4	28	3	2.84	0.43	7	0.0	18	74.1
26	QR	16 July 2009	0.51	40	2.6	20	1	1.00	0.75	18	40.2	12	0.0
28	QR	17 July 2009	1.00	195	6.7	34	3	2.95	0.42	18	0.0	18	87.1
32	QR	18 July 2009	0.63	49	2.1	17	3	2.75	0.43	20	0.0	17	8.0
2	QL	10 July 2006		163	7.5	49	1	1.00	0.75				
4	QL	25 July 2006		44	9.1	38	1	1.00	0.75				
15	QL	10 July 2009		273	12.9	59	3	2.30	0.47				
18	QL	12 July 2009		111	2.7	25	2	1.92	0.51				
19	QL	12 July 2009		158	5.1	27	3	2.76	0.43				
20	QL	13 July 2009		152	15.1	44	1	1.00	0.75				
21	QL	13 July 2009		121	9.2	33	1	1.00	0.75				
24	QL	15 July 2009		50	14.0	27	3	1.25	0.65				
27	QL	16 July 2009		19	0.0	19	1	1.00	0.75				
30	QL	17 July 2009		26	8.7	22	1	1.00	0.75				
31	QL	18 July 2009		67	3.0	22	2	1.94	0.51				
33	QL	19 July 2009		64	10.9	27	2	1.80	0.53				
Mean			0.76*	113*	7.4*	32*	2*	1.46†	0.59*	19*	10.0*	18*	56.8*

*Arithmetic mean.
†Harmonic mean.

with VIC, PET and FAM (Applied Biosystems). The PCR cycle consisted of an initial denaturation at 95 °C for 15 min followed by 30 cycles of 30 s at 95 °C, 90 s at 50 °C and 60 s at 72 °C and a final extension of 30 min at 60 °C. Genotypes were assessed by comparison with internal Genescan 500 LIZ size standards (Applied Biosystems) using an ABI-3130 Avant capillary sequencer. Allele sizes were scored using the supplied Gene Mapper software (Applied Biosystems) and checked by eye.

Queen mating frequency

To determine the relatedness between workers in each colony, 17–71 (mean = 32) workers and worker pupae, including all reproductive workers (cf. section on Worker reproduction and male parentage below), were genotyped. Genotypes of the mother queens were determined by direct genotyping (for the queenright colonies) or determined indirectly from the genotypes of her worker offspring (for the queenless colonies) using the program Colony2 (Wang 2004; Jones & Wang 2010). Workers that did not share an allele with the queen at a given locus were regarded as anomalous and were excluded when assigning individual workers to particular patrilines using Colony2. Individual patriline assignments were then used to calculate the effective queen mating frequency for each colony (Starr 1984), $M_e = 1/\sum p_i^2$, where p_i is the proportional contribution of the ith male mate. The corresponding pedigree worker–worker relatedness was calculated for each colony as $r = 1/4 + 1/2M_e$ (Pamilo 1991a). Arithmetic mean relatedness and the corresponding harmonic mean paternity were calculated as population summary values. The harmonic mean is used because pedigree relatedness and paternity are inversely related (Boomsma 1996). Two types of errors potentially confound effective paternity estimates: nondetection and nonsampling (Boomsma 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 of the patrilines present are not sampled (Foster & Ratnieks 2001a; Nielsen *et al.* 2003). The magnitude of both errors was evaluated using the equations given in Foster & Ratnieks (2001a).

Worker reproduction and male parentage

To determine the number of reproductive workers in each colony, all workers present at the time of collection were dissected. Reproductive workers were defined as those having activated ovaries, i.e. containing at least one oocyte larger than half the size of a freshly worker-laid egg. From this, the proportion of eggs that were laid by workers in each queenright colony was estimated as

$$A = (n_W \cdot p \cdot e_W)/(n_W \cdot p \cdot e_W + e_Q),$$

where n_W is the colony size, p the proportion of reproductive workers and e_W and e_Q the number of eggs laid by a single worker and by the queen per day. The average number of eggs laid by a single worker and by the queen per day in *D. saxonica* is 1.53 and 7.50, respectively (Foster & Ratnieks 2000; K. R. Foster, personal communication). In this calculation, we assume that only the proportion of workers with activated ovaries might change over the course of the colony cycle, but that the fecundity of individual reproductive workers remains more or less constant. From A, we calculated the proportion of male eggs that were worker-laid in a queenright colony as

$$E = A/(A + (1 - A) \cdot m),$$

where A is the proportion of eggs that were laid by workers and m is the proportion of the queen-laid eggs that are male. As the precise sex ratio of queen-laid eggs is unknown, three estimates of E were calculated: E_1, E_2 and E_3 for $m = 0.33$, 0.5 and 0.66, respectively.

To determine the proportion of adult males that were worker produced in queenright colonies, 19–54 (mean = 38) males per colony were genotyped (Table 1), consisting of pupae collected from small and large cells (from the first and second comb with large cells). The proportion of worker-produced males (WPM) for each category (pupae from large cells and pupae from small cells) in each nest was estimated as $N_w/(P_jN_j)$, where N_j and N_w are the total number of males analysed and the number of detected worker's sons for the jth nest, respectively, and P_j is the probability of detecting a worker's son in the jth nest, which was calculated following Foster & Ratnieks (2001). The overall proportion of worker-produced males per nest (Table 2) was calculated as the average of the proportion of worker-produced sons in large and small cells. From these parameters, the proportion of worker-laid eggs that were eaten were then calculated as

$$D = (E - \text{WPM})/E,$$

where E is the proportion of male eggs that were worker-laid and WPM is the proportion of worker-produced males. Following Wenseleers & Ratnieks (2006b), the effectiveness of policing P was calculated as one minus the relative survival of worker-laid eggs, which is given by

$$P = 1 - ((\text{WPM}/E) \cdot (1 - E)/(1 - \text{WPM})),$$

where E is the proportion of male eggs that were worker-laid and WPM is the proportion of worker-produced males.

Table 2 Data on male parentage and the sex ratio in which males and gynes were reared in 11 queenright *Dolichovespula saxonica* colonies. The different columns show the estimated percentages of male eggs laid by workers assuming that the queen laid male and female eggs in a ratio of 1:2 (E_1), 1:1 (E_2) or 2:1 (E_3), the percentage of adult worker-produced males (WPM), the estimated percentages of worker-laid eggs that were eaten, again assuming that the queen laid male and female eggs in a ratio of 1:2 (D_1), 1:1 (D_2) or 2:1 (D_3), the numerical sex ratio and the investment sex ratio

Colony	S	Effective queen mating frequency	Pedigree sister–sister relatedness	E_1 (%)	E_2 (%)	E_3 (%)	WPM (%)	D_1 (%)	D_2 (%)	D_3 (%)	Numerical sex ratio	Investment sex ratio
1	0.73	2.77	0.43	83.3	76.7	71.4	49.4	40.8	35.7	30.9	0.30	0.50
3	0.64	1.00	0.75	65.0	55.0	48.1	0.0	100.0	100.0	100.0	0.50	0.70
7	0.88	1.60	0.56	83.2	76.5	71.2	42.1	49.4	45.0	40.9	0.11	0.22
16	0.78	2.85	0.43	81.3	74.2	68.5	42.2	48.1	43.1	38.4	0.70	0.86
17	0.71	2.00	0.50	72.9	64.0	57.3	30.6	58.0	52.2	46.7	0.75	0.90
22	0.87	1.54	0.58	89.6	85.1	81.2	48.6	45.8	42.9	40.2	0.62	0.82
23	0.89	1.00	0.75	91.0	87.0	83.5	50.0	45.1	42.5	40.1	0.53	0.75
25	0.71	2.84	0.43	78.8	71.0	65.0	37.0	53.0	47.8	43.0	0.06	0.13
26	0.51	1.00	0.75	38.8	29.5	24.1	0.0	100.0	100.0	100.0	0.81	0.92
28	1.00	2.95	0.42	88.9	84.1	80.1	63.6	28.5	24.4	20.5	0.53	0.74
32	0.63	2.75	0.43	39.2	29.8	24.4	4.2	89.4	86.0	82.9	0.56	0.76
Mean	0.76*	1.68†	0.55*	73.8*	66.6*	61.3*	33.4*	59.8*	56.3*	53.1*	0.50*	0.66*

*Arithmetic mean.

†Harmonic mean.

Because, as previously mentioned, the precise sex ratio of queen-laid eggs is unknown, three estimates of D and P were calculated. The overall effectiveness of policing for this species was calculated as the population average of the three estimates for P.

Worker cuticular hydrocarbon profiles

To determine whether the workers had sufficient information from the workers' cuticular hydrocarbon profiles regarding the mating frequency of their mother queen, we investigated the cuticular hydrocarbon profiles of 33–40 workers for each queenright colony using gas chromatography. Workers were killed by freezing at −20 °C, and cuticular hydrocarbons were extracted by immersing individual workers in 1 mL HPLC-grade pentane (Sigma–Aldrich, Denmark) for 10 min, of which the first and the last 15 s involved gentle mixing. The solvent was then allowed to evaporate at room temperature in a laminar flow cabinet. The extract was resuspended in 100 μL pentane, of which 2 μL were injected splitless in an Agilent 6890N gas chromatograph, equipped with an HP-5MS capillary column (30 m × 250 μm × 0.25 μm), a split–splitless injector, a flame ionization detector and a helium carrier gas flow of 1 mL/min. After an initial hold of 1 min at 70 °C, the temperature was raised to 210 °C at a rate of 30 °C/min, then to 280 °C at 3 °C/min and then to 320 °C again at 30 °C/min, with a final hold at 320 °C of 5 min. Compounds were identified using coupled gas chromatography—mass spectrometry, using an identical Agilent 6890N GC with the same temperature program and a single quadropole Agilent 5375 MS with electron ionization (70 eV), and by subsequently comparing diagnostic ions of the mass spectra with published data (e.g. Bonavita-Cougourdan *et al.* 1987; Howard *et al.* 2001). Peak areas of 56 hydrocarbons (or mixtures of several coeluting hydrocarbons, Fig. S1, Supporting information) found on the cuticles of all workers and queens were quantified using the software Agilent ChemStation (v. D.02.00.237) and normalized with a Z-transformation (Aitchison 1986) using the geometric mean of these 56 compounds. To quantify hydrocarbon variability within colonies, we calculated the average Euclidean distance of workers to their colony centroid, i.e. the square root of the sum of 56 squared differences between the normalized hydrocarbon concentrations of each individual and the colony mean. Hydrocarbon variability was calculated both including and excluding the profiles of reproductive workers (those with activated ovaries, see below). To determine whether worker patrilines could be discriminated within colonies based on their hydrocarbon profile, we first performed a principal component analysis for each colony using the program LatentiX 2.00 (MathWorks Inc., Denmark), after which the scores for principal components with an eigenvalue >1 were used in a discriminant analysis (DA) in the program STATISTICA 7.1 (StatSoft Inc., USA) (Table 3).

Table 3 Data on the amount of information on queen mating frequency encoded in worker cuticular hydrocarbon profiles in 11 queenright *Dolichovespula saxonica* colonies. The different columns show the colony number, absolute queen mating frequency, hydrocarbon variability (including and excluding reproductive workers) and the significance of patriline discrimination based on a DA analysis

Colony	Queen mating frequency	Hydrocarbon variability		Patriline discrimination
		All workers	Without reproductive workers	
1	3	1.66	1.48	$\lambda = 0.017$, $F_{18,48} = 17.56$, $P < 0.001$
3	1	1.91	1.83	—
7	2	1.56	1.41	$\lambda = 0.399$, $F_{6,31} = 7.78$, $P < 0.001$
16	3	1.51	1.43	$\lambda = 0.113$, $F_{16,20} = 2.47$, $P < 0.028$
17	2	1.41	1.28	$\lambda = 0.156$, $F_{7,12} = 9.30$, $P < 0.001$
22	2	1.97	1.37	$\lambda = 0.877$, $F_{7,19} = 0.38$, $P < 0.903$
23	1	1.79	1.39	—
25	3	1.49	1.48	$\lambda = 0.035$, $F_{18,18} = 4.34$, $P < 0.002$
26	1	1.43	1.38	—
28	3	1.22	1.22	$\lambda = 0.033$, $F_{16,16} = 4.50$, $P < 0.003$
32	3	2.06	1.97	$\lambda = 0.091$, $F_{16,12} = 1.74$, $P < 0.169$

Parameters explaining variation in male parentage and sex ratio

To test for the presence of facultative adjustment of levels of worker policing (Foster & Ratnieks 2000) or worker reproduction (Wenseleers *et al.* 2004) according to the colony kin structure, we checked whether male parentage (WPM, E_1, E_2, E_3, D_1, D_2 and D_3) correlated with worker–worker relatedness or absolute or effective queen mating frequency using Spearman's rank correlations. In addition, we checked whether male parentage (WPM, E_1, E_2, E_3) correlated with worker–worker relatedness after correcting for the influence of the stage of colony development S using Spearman's rank correlations. Wilcoxon matched pairs tests and Mann–Whitney U tests were used to test for differences between the percentage of males that were workers' sons in the egg vs. in the adult stage (E_1, E_2 and E_3 vs. WPM) and the inferred percentage of worker-laid eggs that were eaten (D_1, D_2 and D_3) in colonies headed by a single-mated vs. a multiple-mated queen. To determine which factors best explained the variation in the percentage of adult males that were workers' sons, we used a forward stepwise regression model (P to enter 0.05, P to remove 0.05) with the following continuous predictor variables: collection date, colony size (number of adult workers present), stage of colony development S, number of reproductive workers, worker–worker relatedness and hydrocarbon variability (both including and excluding reproductive workers, Table 3). Given that the relationship between the percentage of male eggs and adult males that were workers' sons and the parameter S was sigmoidal, a

logistic function $y = k/(1 + e^{-b \cdot (x-m)})$ was fitted in bivariate scatter plots, with k being the upper asymptote, b the growth rate and m the time of maximal growth.

Spearman's rank correlations between investment sex ratio and the parameter S, worker–worker relatedness, absolute queen mating frequency and effective queen mating frequency were used to determine whether colony sex ratios in queenright colonies were split according to queen mating frequency (Boomsma & Grafen 1990; Sundström 1994; Sundström *et al.* 1996; Meunier *et al.* 2008; West 2009) or stage of colony development. In addition, one-sample *t*-tests were used to compare observed investment sex ratios to those predicted under either queen or worker control. Irrespective of the level of worker reproduction in a queenright colony, the investment sex ratio under queen control (expressed as female investment) is expected to be 0.5, whereas the investment sex ratio expected under worker control is given by

$$\frac{r}{r + ((1 - \psi) \cdot 0.5 + \psi \cdot r)/(2 - \psi)},$$

where r is the pedigree sister–sister relatedness and ψ is the proportion of males produced by workers in queenright colonies (Pamilo 1991b). When workers not only reproduce in queenright colonies but also produce a proportion β of the males in orphaned colonies, the expected investment sex ratio under queen control is given by

$$\frac{0.5}{0.5 + ((1 - \beta) \cdot (1 - \psi) + (1 - \beta) \cdot \psi \cdot 0.5)/(1 + (1 - \beta) \cdot (1 - \psi))},$$

whereas the expected investment sex ratio under worker control is given by

$$\frac{r}{r + ((1 - \beta \cdot (1 - \psi) \cdot 0.5 + (1 - \beta) \cdot \psi \cdot r)/}{(1 + (1 - \beta) \cdot (1 - \psi))}\,,$$

where r is the pedigree sister–sister relatedness, ψ the proportion of males produced by workers in queenright colonies and β the proportion of males produced in orphaned colonies (Pamilo 1991b). All statistical analyses were carried out in STATISTICA 9.1 (Statsoft Inc.).

Results

Queen mating frequency

Genetic variation at the three microsatellite loci studied was high, with 15, 20 and 8 detected alleles and expected heterozygosities of 0.90, 0.85 and 0.75 at loci *Rufa05*, *Rufa13* and *Rufa18*, respectively. The arithmetic average queen mating frequency was 2.0 (95% CI = 1.6–2.3), with 39% of the queens being single mated and the remainder being either double or triple mated (Table 1). The harmonic mean effective queen mating frequency, taking into account unequal paternity contributions, was 1.46 (95% CI = 1.24–1.78), resulting in an average worker–worker pedigree relatedness of 0.59 (95% CI = 0.53–0.65) (Table 1). The harmonic mean effective queen mating frequency in colonies headed by a multiple-mated queen was 2.08 ($N = 14$), which is very close to the threshold of 2, above which worker policing is selected for on grounds of relatedness, as workers in such colonies are collectively more related to the queen's sons than to workers' sons. In comparison, the harmonic mean effective queen mating frequency for polyandrous queens in the British population studied by Foster & Ratnieks (2000) was 1.74 ($N = 5$). Our paternity estimates were robust, given that the nondetection error was very low, 0.003, and that, with an average of 32 workers per colony being genotyped, the mean nonsampling probabilities of males with 50%, 33% or 25% paternity were only 2.3×10^{-10}, 2.7×10^{-6} and 1.0×10^{-4}, on average. Hence, neither of these two errors significantly affected our paternity estimates.

Only 18 of 718 (2.5%) genotyped workers had anomalous genotypes, i.e. genotypes that could not be assigned to the majority matriline. Two-thirds of these anomalous genotypes were found in queenless colonies (12/18 in the four queenless colonies 2, 8, 27 and 30; 6/18 in the four queenright colonies 3, 22, 25 and 28). When extrapolating the percentage of anomalous worker genotypes found in the subset of genotyped workers, workers with an anomalous genotype made up only a small proportion of the total work force (3.1–8.1%, mean = 5.0%), except for queenless colony 19, where 25.8% workers had an anomalous genotype. In all colonies in which multiple workers with an anomalous genotype were found could such workers be assigned to a single matriline. This implies they were most likely the offspring of an usurped queen as opposed to workers that had drifted from another colony and hence provides evidence for the occurrence of nest usurpation during the founding stage or possibly following the death of the original founding queen in *D. saxonica* (Greene 1991; Foster *et al.* 2001). In the four queenright and four queenless colonies where anomalous worker genotypes were found, the percentage of workers with anomalous genotypes having activated ovaries (9.7% in queenright colonies and 6.8% in queenless colonies) was not significantly different from that found among the workers with a regular genotype (10.0% in queenright colonies and 6.6% in queenless colonies) (Wilcoxon matched pairs tests, $P = 0.72$ for queenright colonies and $P = 1.0$ for queenless colonies). Despite the significant percentage of workers with anomalous genotypes that had activated ovaries, there was no evidence that they had produced any of the genotyped males.

Worker cuticular hydrocarbon profiles

The 56 cuticular hydrocarbons consisted of linear alkanes and alkenes and branched alkanes with a chain length ranging from C_{23} to C_{31} (Fig. S1, Supporting information). Patrilines could be significantly discriminated based on cuticular hydrocarbon profiles in 6 of 8 (75%) of the colonies with multiple patrilines (Table 3). Nevertheless, this still implies that in 25% of the cases, the workers lacked accurate information on queen mating frequency and hence would end up making a mistake, e.g. by not policing worker reproduction even when it would be beneficial to do so, which would encompass a significant cost. In addition, even when mating frequency was coded as a discontinuous trait (i.e. as monandrous or polyandrous), it still did not correlate with hydrocarbon variability (Spearman's rank correlation, hydrocarbon variability including reproductives, $R = -0.13$, $P = 0.71$; excluding reproductives, $R = -0.06$, $P = 0.85$). Furthermore, hydrocarbon variability did not correlate with relatedness or absolute or effective queen mating frequency (Spearman's rank correlation, hydrocarbon variability including reproductives, $-0.40 < R < 0.40$, $P > 0.22$ for all three R values; excluding reproductives, $-0.014 < R < 0.20$, $P > 0.55$ for all three R values). Hence, worker cuticular hydrocarbon profiles encoded only a limited amount of information on queen mating frequency.

Worker reproduction and male parentage

Overall, 6.5% ($N = 1188$) and 8.2% ($N = 1237$) of the dissected workers had activated ovaries in queenright and queenless colonies, respectively. This difference was not statistically significant, given that the presence or absence of a queen, relatedness, or the interaction between both factors did not have a significant effect on the percentage of workers with activated ovaries (ANCO-VA, presence of the queen: $F_1 = 0.03$, $P = 0.86$; relatedness: $F_1 = 0.47$, $P = 0.50$; queen presence × relatedness: $F_1 = 0.005$, $P = 0.94$). This pattern is in line with previous dissection data for *D. saxonica* (Foster & Ratnieks 2000; Foster *et al.* 2001; Wenseleers & Ratnieks 2006b), showing that the percentage of workers with activated ovaries does not increase after the loss of the mother queen and that workers do not facultative change their rates of ovary activation in response to queen mating frequency. The percentage of workers with activated ovaries, however, did correlate with the stage of colony development S (Spearman's rank correlation, $R = 0.77$, $P = 0.006$). Based on the observed percentage of workers with active ovaries per colony and the fact that workers and the queen on average lay 1.53 and 7.50 eggs per day, respectively (Foster & Ratnieks 2000; K. R. Foster, personal communication), the percentage of eggs that were laid by workers in queenright colonies was estimated to vary between 17.3% and 77.0% (mean = 52.9%). The average percentage of male eggs laid by workers in queenright colonies was estimated at 61.3%, 66.6% or 73.8%, depending on whether the queen was assumed to lay male and female eggs in a ratio of 1:2 (E_1), 1:1 (E_2) or 2:1 (E_3) (Table 2). These estimates are probably quite accurate, given that the percentage of workers with active ovaries is perfectly in line with the actual observed number of workers that lay eggs in the sister species *Dolichovespula norwegica* (Bonckaert *et al.* 2010) and that unlike ants and stingless bees, wasps are not known to lay unviable trophic eggs. Large differences in the relative fecundities of queens and reproductive workers relative to the earlier studied British population of *D. saxonica* are also unlikely, given that the relative fecundity of a queen to a worker in the closely related species *Dolichovespula sylvestris* was very close to our estimate for *D. saxonica*, 4.8 (T. Wenseleers, unpublished data) and that in *D. sylvestris*, the number of laying workers also strongly correlates with the percentage of eggs laid by workers (Pearson's correlation, $r = 0.78$, $P = 0.023$, $n = 8$ colonies; Wenseleers *et al.* 2005; T. Wenseleers, unpublished data). Furthermore, we think that it is unlikely that the workers' egg-laying rate would covary with colony kin structure (queen mating frequency), given that there was no correlation between the average worker egg-laying rate and queen mating frequency in the vespine wasps *D. sylvestris* (Spearman's correlation, $R = -0.16$, $P = 0.73$, $N = 7$, T. Wenseleers, unpublished data) and *Vespula germanica* (Spearman's correlation, $R = -0.50$, $P = 0.67$, $N = 3$, Bonckaert *et al.* 2008).

In contrast to the high percentages of male eggs that were estimated to be worker-laid, the genotyping results show that only 33.4% of the adult males in queenright colonies were workers' sons (Table 2). This means that 53.1–59.8% of the worker-laid eggs were eaten in queenright colonies, resulting in an estimated effectiveness of policing of 75.9–88.0%, a range that encloses the effectiveness of policing found in a British population of *D. saxonica*, 87.5% (Wenseleers & Ratnieks 2006b).

Parameters explaining male parentage

The estimated percentage of male eggs laid by workers (E_1, E_2 and E_3), the percentage of adult males that were workers' sons (WPM) and the estimated percentage of worker-laid eggs that were eaten (D_1, D_2 and D_3) in the 11 queenright colonies did not show any significant correlation with either relatedness, absolute queen mating frequency or effective queen mating frequency (Spearman's rank correlation, $-0.60 < R < 0.60$, $P > 0.052$ for all 21 R values). The same conclusion was reached when the influence of the stage of colony development S was first corrected for (which was found to have a large influence on male parentage, see below), as there was no correlation between the residuals in the level of worker reproduction (E_1, E_2, E_3 and WPM) relative to the best-fit sigmoidal influence of stage colony S (Fig. 2) and relatedness (Spearman's rank correlation, $-0.39 < R < 0.34$, $P > 0.24$ for all four R values). Hence, in contrast to the earlier study by Foster & Ratnieks (2000) on a British population of *D. saxonica*, there was no evidence that the workers in our Danish study population facultatively policed worker-laid eggs only in colonies headed by a multiple-mated queen. Low power cannot account for this result given that Foster & Ratnieks (2000) reported a Pearson's correlation r between worker–worker pedigree relatedness and the percentage of adult males that were workers' sons of 0.82 for the British population ($N = 9$; K. R. Foster, personal communication) and that we had a power of 0.96 to detect such a large correlation if it would have been present in the Danish one (using a one-sided test), given our study of 11 queenright colonies and use of an alpha significance cut-off of 0.05. In fact, even if we pool our data on the colony kin structure and levels of worker reproduction in *D. saxonica* with those obtained by Foster & Ratnieks (2000), the percentage of adult males that were worker produced still did not correlate with relatedness

or effective queen mating frequency (Spearman's rank correlation, $R = -0.02$, $P = 0.94$ and $R = -0.23$, $P = 0.82$, $N = 20$).

In terms of levels of policing, there was no difference in the estimated percentage of worker-laid eggs that were eaten in colonies headed by a single- or a multiple-mated queen (Mann–Whitney U tests, $P > 0.18$ for all three D values). Nevertheless, the percentage of adult males that were workers' sons (33.4%) was significantly lower than the estimated percentage of male eggs that were laid by workers (61.3–73.8%, Table 2) (Wilcoxon matched pairs tests, E_1, E_2 and E_3 vs. WPM, one-sided $P = 0.002$ for all three tests). This shows that worker-laid eggs were policed, although not facultatively in response to queen mating frequency. Interestingly enough, the estimated percentage of worker-laid eggs that were eaten (D_1, D_2 and D_3) correlated negatively with the stage of colony development S (Spearman's rank correlation, $R < -0.83$, $P < 0.002$ for all three R values), suggesting that early in the colony cycle relatively more worker-laid eggs were policed. An alternative explanation, though, which we cannot exclude, could be that the egg-laying rate of reproductive workers was lower in the beginning of colony development and that our estimates of the policing effectiveness were biased by this. A forward stepwise regression model showed that the only parameter that explained a significant amount of variation in the percentage of adult males that were workers' sons was not relatedness (Fig. 1), but the stage of colony development S ($P = 0.001$, both P to enter and P to remove; Fig. 2). The same result was obtained after rank transforming all variables, or when the two colonies in which the hydrocarbon profiles indicated that the workers were unable to discriminate among the different patrilines (Table 3) were excluded. In bivariate plots, the relationship between the level of worker reproduction (E_1, E_2, E_3 and WPM) and stage of colony development (S) showed a sigmoid relationship and a logistic fit resulted in R^2 values of 0.85, 0.87, 0.88 and 0.93, respectively (Fig. 2).

Lastly, an interesting result with respect to levels of worker reproduction in queenright colonies was that significantly more WPM were found among the males reared in the upper, small-celled comb (mean = 56.8%) as opposed to in the lower, large-celled combs (mean = 10.0%) (Mann–Whitney U test, $P = 0.007$). This means that levels of worker reproduction varied significantly depending on the location in which the male brood was reared.

Investment sex ratio

With 33.4% of the males in queenright colonies being workers' sons, the expected investment sex ratio under
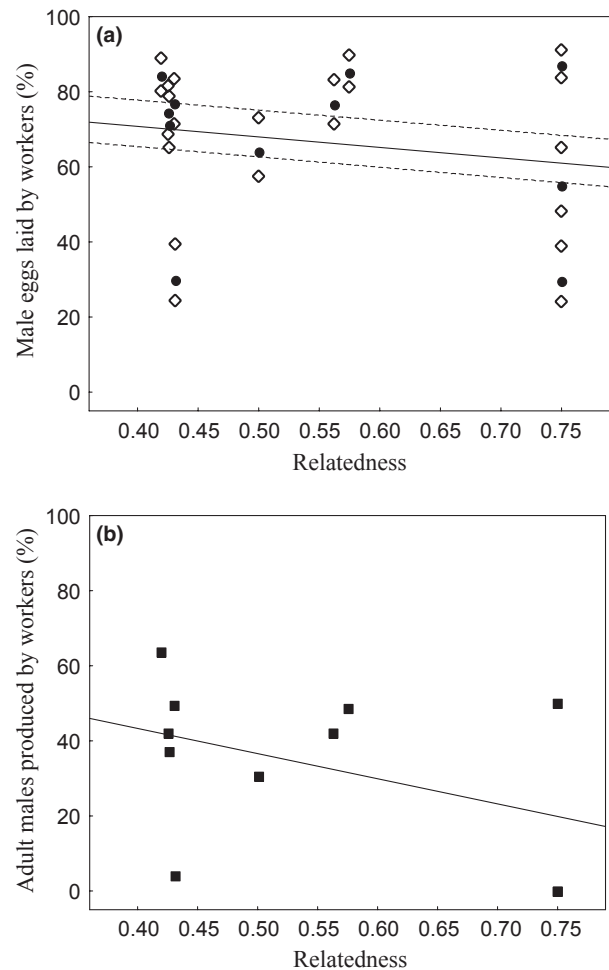

Fig. 1 Estimated percentage of male eggs that are laid by workers (a) and percentage of adult males that are workers' sons (b) as a function of worker–worker pedigree relatedness, for 11 queenright *Dolichovespula saxonica* colonies (a linear fit is shown for illustrative purposes). In panel a, three estimates are given for each colony, corresponding to an assumed male to female sex ratio of queen-laid eggs of 1:2 (E_1, top ◇), 1:1 (E_2, •) and 2:1 (E_3, bottom ◇). Estimates of the parentage of adult males were based on the genotyping of an average of 18 and 19 male pupae from small-celled and large-celled combs, respectively.

queen and worker control was 0.5 and 0.64, respectively. The observed investment sex ratio in queenright colonies was 0.66, which was not significantly different from the one expected under worker control (one-sample t-test, $t_{10} = 0.31$, $P = 0.76$), and more female biased than expected if the sex ratio was under control of the queen (one-sample t-test, $t_{10} = 2.03$, one-sided $P = 0.035$). The same conclusion held if one took into account that workers not only reproduced in queenright colonies but also produced a certain fraction of the males in orphaned nests. In particular, if workers
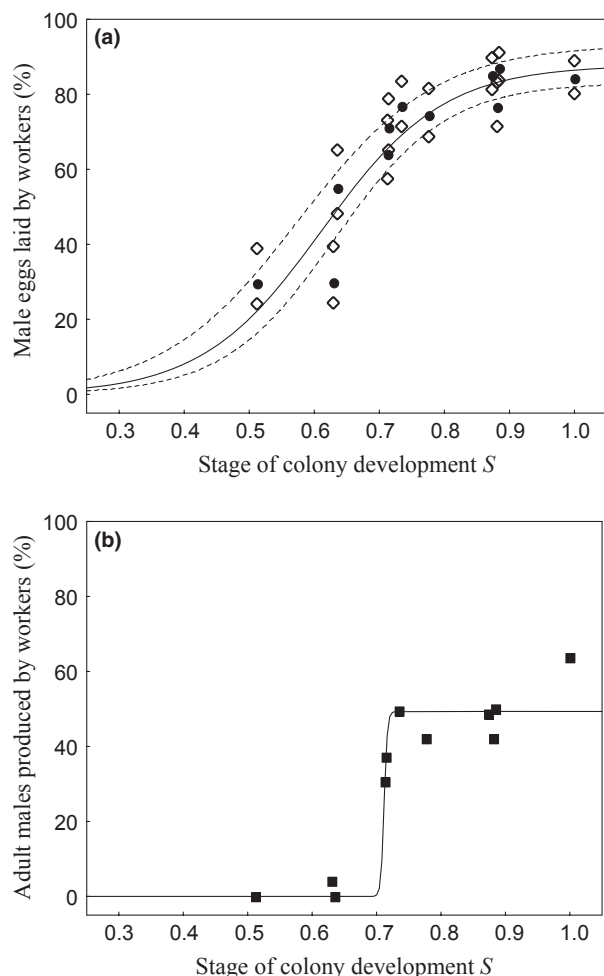
Fig. 2 Estimated percentage of male eggs that are laid by workers (a) and percentage of adult males that are workers' sons (b) as a function of the stage of colony development S, for 11 queenright *Dolichovespula saxonica* colonies (a sigmoid logistic fit is shown for illustrative purposes). In panel a, three estimates are given for each colony, corresponding to an assumed male to female sex ratio of queen-laid eggs of 1:2 (E_1, top ◇), 1:1 (E_2, •) and 2:1 (E_3, bottom ◇). Estimates of the parentage of adult males were based on the genotyping of an average of 18 and 19 male pupae from small-celled and large-celled combs, respectively.

produced up to 10% of the males in orphaned nests, the investment sex ratio in our queenright colonies was still more female biased than that expected under queen control (one-sample *t*-test, $t_{10} = 1.83$, one-sided $P = 0.049$) and did not differ from the expected investment sex ratio under worker control (one-sample *t*-test, $t_{10} = 0.12$, $P = 0.90$); if more than 10% of the males were produced by workers in orphaned nests, the observed investment sex ratio in queenright colonies would become intermediate between that expected under queen and worker control (one-sample *t*-tests, $t_{10} = 1.81$,

one-sided $P = 0.050$ and $t_{10} = -0.20$, one-sided $P = 0.42$, respectively). Importantly, and further reinforcing the idea that there was no facultative adjustment of the workers' behaviour in function of relatedness, the investment sex ratio did not correlate with relatedness, absolute queen mating frequency or effective queen mating frequency, and it also did not correlate with the stage of colony development S (Spearman's rank correlation, $-0.33 < R < 0.23$, $P > 0.33$ for all four R values).

Discussion

Our results demonstrate that worker reproduction in a Danish population of the Saxon wasp *Dolichovespula saxonica* was not correlated with queen mating frequency, unlike what seemed to be the case in an earlier study of a British population of the same species (Foster & Ratnieks 2000). Instead, our study shows levels of worker reproduction to strongly correlate with colony development and the location in the nest where the males were reared. In addition, we showed that the percentage of adult males that were workers' sons, 33.4%, was significantly lower than the percentage of male eggs that were estimated to be laid by workers, 61.3–73.8%. This means that even though workers do not facultatively adjust their policing behaviour in response to the queen mating frequency, queen and/or worker policing was present in all queenright colonies. This is in line with data from other species of *Dolichovespula* where worker reproduction is policed both by the queen and by reproductive and nonreproductive workers (Foster & Ratnieks 2001b; Wenseleers *et al.* 2005; Wenseleers & Ratnieks 2006a; Bonckaert *et al.* 2010). In addition, there was evidence that the estimated percentage of worker-laid eggs eaten was highest early on in the colony cycle and that it subsequently decreased.

That worker reproduction increases and the effectiveness of the policing decreases as the season progresses is a significant finding given that our data are the first to show such a pattern in Vespinae wasps. A similar pattern, however, is found in another group of social insects with an annual life cycle, the bumblebees, where the 'competition point', which is defined as the start of worker reproduction, comes relatively late in the colony cycle (Duchateau & Velthuis 1988; Bloch & Hefetz 1999; Bourke & Ratnieks 2001). The pattern we document is also in line with a recent theoretical model by Ohtsuki & Tsuji (2009), which predicts that there should be less worker reproduction and more worker policing in the early ergonomic stages of the colony growth, as worker reproduction at an early stage would greatly hinder the growth of the work force and therefore come at a cost to the workers' future inclusive fitness. Our last novel finding on worker reproduction was that male

parentage was dependent on the location in which the males were reared and that relatively more workers' sons were reared in the upper small-celled comb as opposed to in the lower large-celled combs. This could be because of the queen spending more time on the large-celled sexual combs in order to lay female, gyne-destined eggs, thereby giving her more opportunity to dominate egg laying as well as to police worker-laid eggs in this part of the nest. Alternatively, it might also be expected to be less beneficial for the workers to lay their male-destined eggs in large-celled sexual combs, as the production of males in these cells would interfere with the production of highly related sister gynes (Foster & Ratnieks 2001b; Wenseleers & Ratnieks 2006a).

In further support of the idea that workers did not facultatively adjust their behaviour according to the colony's relatedness structure and might not even have had the means to do so, we found that variability in cuticular hydrocarbon profiles among the workers did not significantly correlate with relatedness and queen mating frequency. In addition, in one-quarter of the multiple-mated colonies, patrilines could not be discriminated based on the workers' cuticular hydrocarbon profiles. Hence, the workers in our study population probably did not have sufficient information on the queen mating frequency, based on variation in the workers' cuticular hydrocarbon profiles, to facultatively police worker-laid eggs only in nests headed by multiple-mated queens. Earlier, Dani *et al.* (2004) also showed cuticular hydrocarbons to contain only limited information for discriminating full-sisters and half-sisters in another vespine wasp, the hornet *Vespa crabro*.

There was also no evidence that workers facultatively policed worker-laid eggs only in colonies where they had accurate information on queen mating frequency, since even after exclusion of two colonies in which the hydrocarbon profiles indicated that the workers were unable to discriminate among the different patrilines, there was no significant correlation between worker–worker relatedness and the percentage of adult males that were worker produced. This was unlike a study on the wood ant *Formica truncorum*, where errors in facultative sex ratio biasing were shown to be owing to informational constraints in the workers' hydrocarbon profiles (Boomsma *et al.* 2003). In fact, in the context of sex ratio biasing in our study, there was also no evidence for sex ratios being split and adjusted in response to the queen mating frequency and the relatedness asymmetry of males vs. females (Boomsma & Grafen 1990; Sundström 1994; Sundström *et al.* 1996; Meunier *et al.* 2008; West 2009). This further supports our idea that workers do not facultatively adjust their behaviour in response to the queen mating frequency in this species. Interestingly, however, our data showed that

overall, the investment sex ratio was female biased and that it was probably under the control of the workers. This is the first formal evidence for possible worker control of the investment sex ratio in Vespinae wasps, in contrast to earlier data from the vespine wasp *Vespula maculifrons* where the population sex ratio did not significantly differ from either the queen or worker optimum (Johnson *et al.* 2009). Tentative evidence for female-biased investment sex ratios was also presented for other species of Vespinae wasps in Foster & Ratnieks (2001b) and for *Polistes* paper wasps in Suzuki (1986).

Overall, our results suggest that earlier evidence for facultative worker policing in *D. saxonica* (Foster & Ratnieks 2000) may have been due to accidental correlations with possible confounding variables, such as the colony stage of the sampled nests or the location inside the nest in which the males were reared, which were not accounted for in the original study. Alternatively, it might be the case that there are large interpopulation differences in the expression of worker policing, for example, due to varying informational constraints on queen mating frequency. More generally, our results add credence to the idea that workers in eusocial Hymenoptera primarily respond to population-level averages of kin parameters and that facultative adjustment of behaviour in response to the colony-specific kin structure may be relatively rare. Earlier, this was also suggested to be the case by the fact that predictions of inclusive fitness theory in eusocial Hymenoptera were in general well supported interspecifically (e.g. Wenseleers & Ratnieks 2006a) but not in the context of intraspecific comparisons (e.g. Hammond *et al.* 2003; Bourke 2005; Wenseleers & Ratnieks 2006a). Hence, our results by no means imply that inclusive fitness theory is wrong or somehow limited in scope, but rather that studies have to take into account proximate constraints as well as other ecological factors affecting fitness costs and benefits.

Acknowledgements

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Data Accessibility

Microsatellite data, cuticular hydrocarbon data and surface area of the combs: Dryad entry doi:10.5061/dryad.9pd1q.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Typical gas chromatogram of the cuticular hydrocarbon profile of a nonreproductive *Dolichovespula saxonica* worker.

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