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Worker drift and egg dumping by queens in wild *Bombus terrestris* colonies

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Abstract Wild bumblebee colonies are hard to find and often inaccessible, so there have been few studies of the genetic structure of bumblebees within natural colonies, and hence, it is not clear how frequently events such as worker reproduction, worker drift and queen usurpation take place. This study aimed to quantify the occurrence of natal-worker reproduction, worker drift and drifter reproduction within 14 wild colonies of *Bombus terrestris* in Central Scotland. Four unlinked microsatellites were used to identify patterns of relatedness of the colonies' adults and broods. In colonies with queens (queenright colonies), worker reproduction accounted for just 0.83 % of males, increasing to 12.11 % in queenless colonies. Four colonies contained a total of six workers which were not daughters of the queen, and were assumed to be drifters, and four male offspring of drifters. Drifting is clearly not common and results in few drifter offspring overall, although drifters produced approximately seven times more offspring per capita than workers that remained in their natal colony. Unexpectedly, two colonies contained clusters of sister workers and juvenile offspring that were not sisters to the rest of the adults or brood found in the colonies, demonstrating probable egg dumping by queens. A third colony contained a queen which was not a sister or daughter to the other bees in the colony. Although usurping of bumblebee colonies by queens in early season is well documented, this appears to be the first record of egg dumping, and it remains unclear whether it is being carried out by old queens or newly mated young queens.

Keywords Egg dumping · Drifting · *Bombus* · Colony parasitism

Introduction

In colonies of social hymenopterans, the majority of individuals do not reproduce; instead, they assist with rearing their siblings, some of which will go on to reproduce. It is also possible for workers to reproduce directly by laying unfertilised eggs which develop into males (Sladen 1912; Van Honk et al. 1981; Duchateau 1989; Bourke and Ratnieks 2001).

Worker bees entering colonies other than their own are known as drifters. Drifting is a well-described phenomenon in *Apis mellifera* and can be extensive, with drifters sometimes comprising more than half of the workforce (Free 1958; Jay 1966; Neumann et al. 2000; Chapman et al. 2010a). Drifting is thought to be largely the result of orientation errors and occasionally due to nectar robbing (Pfeiffer and Crailsheim 1998; Neumann et al. 2000). The layout and external appearance of hives, as well as environmental factors such as the prevailing wind and sun direction, can also affect the likelihood of drifting (Free 1958; Jay 1965, 1966, 1968, 1971). Worker drift and reproduction have been documented in other social hymenopterans, where drifters may act as social parasites entering the colonies and laying eggs. For example, *Halictus ligatus* females drift between nest areas to lay eggs and do not carry pollen or assist with burrowing duties (Packer 1986). Workers of other bees also drift into, and lay eggs in, non-natal colonies, including *Apis cerana* (Nanork et al. 2007), *Melipona scutellaris* (Alves et al. 2009) and *Apis florea* (Nanork et al. 2005; Chapman et al. 2010b).

Drifter bumblebee workers were found in non-natal colonies in an experiment using laboratory-reared colonies of

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Bombus terrestris which were allowed to forage freely (Lopez-Vaamonde et al. 2004). Colony inspections of colour-marked workers confirmed that workers were entering non-natal colonies, with a mean of nine drifters observed per colony. Microsatellite analysis of callow males revealed that of the 1,501 males examined, 28 were produced by resident workers and 53 by drifters. Furthermore, nine of the drifter males were not attributable to any other colonies in the experiment and so must have resulted from drifting from wild bees.

Drifting has been found in glasshouses and agricultural systems where commercially reared bumblebee colonies are used to pollinate crops. In *B. terrestris*, depending on colony orientation, up to 50 % of the workforce can be composed of drifters (Lefebvre and Pierre 2007). For *Bombus occidentalis* and *Bombus impatiens*, an average of 28 % of workers was discovered to be drifters (Birmingham and Winston 2004). The ovaries of drifters are more likely to be developed than those of resident workers (Birmingham et al. 2004). Whilst some experiments that detected drifting workers were carried out at high densities, others were at lower densities, which are not dissimilar from those expected in the field. For example, Birmingham and Winston (2004) studied *B. impatiens* colonies that were stocked at a maximum of seven colonies per hectare, which is comparable to estimates of wild colony densities (Knight et al. 2005, 2009; Osborne et al. 2008).

Most observations of drifting in bumblebees have been in artificial systems where the bees were housed in domiciles with similar appearance and entrances. Wild colonies are typically in the burrows and nests of small mammals (Fussell and Corbet 1992), and the entrances are often hidden amongst vegetation. The entrances of artificial colonies tend to be comparatively short compared with the several metres of tunnels typical of some species such as *B. terrestris* (Sladen 1912; Carvell et al. 2008). It is therefore likely that drifting may not occur in the wild to the same extent as in artificial settings. Drifting has been confirmed at a low level in wild colonies of *Bombus deuteronymous* in Japan, and this remains the only evidence for drifting in natural colonies (Takahashi et al. 2010). Of the 11 colonies examined, 3 contained four drifters in total and eleven of their male offspring. Intraspecific parasitism has also been demonstrated within other hymenopterans. Queenless colonies of *M. scutellaris* may be usurped by foreign queens that take over the colony and lay eggs (Alves et al. 2009). Colony usurpation has been documented in bumblebees and is thought mainly to occur early in the season, before the second brood of workers have emerged (Sladen 1912; Hobbs 1965; Alford 1975). The presence of one or more dead queens in the colony is often the only evidence for usurpation or attempted usurpation by queens (e.g. Carvell et al. 2008). Molecular techniques now allow the identification of foreign queens and reveal the extent to which such queens are successful at reproducing. Usurping has been detected using genetic techniques where it might otherwise have gone unnoticed. For example, in one colony of *Bombus hypnorum* (Paxton et al. 2001), workers in this colony were not all of the same lineage, suggesting usurpation had taken place although drifting could not be ruled out in this case.

Here, we examine patterns of relatedness among the adults and brood of 14 wild, mature colonies of *B. terrestris*, the largest sample of wild bumblebee colonies yet to be examined. We present evidence for drifting, drifter reproduction and egg dumping by queens.

Method

In July, August and September 2009 and 2010, 14 colonies of *B. terrestris* were located by searching, aided by the use of a dog trained to detect bumblebee colonies (Waters et al. 2010; O'Connor et al. 2012). All were from the campus of the University of Stirling (Lat/Long 56° 8.874852″ N, 3° 54.928386″ W) and nearby farmland. The colonies were excavated, and the entire comb was removed and frozen at −18 °C. As far as possible, all adult bees were caught using nets. Colonies were later dissected, removing adult bees, eggs, pupae and larvae, including degraded specimens. A colony was considered queenless if no live queen was collected or if the only queens present appeared to be new gynes (without signs of wear).

DNA was extracted from the thoracic muscle for adults and pupae, whole eggs or a similar sized portion of material from larvae, using HotShot protocol (75 μL of HotShot lysis solution, incubated at 95 °C for 1 h followed by addition of a 75-μL neutraliser solution; Truett et al. 2000) and stored at −18 °C until needed. Where colonies had more than 315 specimens, all adults were genotyped, and a non-random sample of eggs and brood, to a total of 315. The non-random sample avoided those that were obviously degraded and later-stage brood, which appeared to be developing gynes, as these were not likely to be natal-worker or drifter-worker offspring. DNA was amplified using Multiplex PCR Kits (Sigma) at four, labelled, unlinked microsatellite loci: B126, B10, BT09 and BT26 (Estoup et al. 1993, 1995, 1996; Funk et al. 2006). Polymerase chain reaction (PCR) was carried out according to the following protocol: 0.02 μL each of forward and reverse of the four primers, 1 μL Q-solution, 5 μL multiplex mix, 2 μL water and 1 μL DNA. Samples were firstly denatured at 95 °C for 15 min and then subjected to 54 cycles of annealing temperature of 48 °C for 30 s and denaturing of 94 °C for 90 s, then extension at 72 °C for 90 s and a final extension step at 72 °C for 10 min.

PCR product was sent to the DNA Sequencing Service, Dundee, UK where fragment analysis was carried out at 1:50 dilution on an ABI 3730 capillary DNA sequencer, and LIZ500 dye (Applied Biosystems) was used as the size standard. Fragment analysis output was examined using STRand software (Veterinary Genetics Laboratory, University of California at Davis, http://www.vgl.ucdavis.edu/informatics/strand.php).

A negative and positive control was included in each 96-well plate of samples prior to PCR to check for contamination of reagents and consistency of PCR and electrophoresis output. Where electrophoresis output was weak for one or all loci, the samples were amplified a second time and genotyped. Samples with one or more alleles foreign to the colony consensus group (i.e. possible drifters) were rechecked with fresh DNA extractions. The error rate was estimated by amplifying DNA and blindly scoring electrophoresis output from 94 samples including individuals from most colonies and all life stages (eggs, brood and adults). Identical results were obtained at the four loci in all cases. Therefore, genotyping error rates were estimated to be acceptably low.

B. terrestris queens are monandrous (Estoup et al. 1995). This means that for any locus, there is a maximum of three possible alleles that a bee can inherit: two maternally (assuming heterozygosity) and one paternally (assuming the male's allele differs from that of the queen). The parent alleles are easily deduced from examining colony mates, and a drifter is taken to be any diploid individual carrying at least one allele that is not possessed by the queen or her mate. Data were inspected visually for individuals that did not conform to the parentage of colony mates.

A drifter can be misclassified by chance if she shares alleles with the rest of the bees in the colony at all four microsatellite loci. The probability of missing a drifter can be estimated by calculating the frequency of the paternal and maternal alleles in the population (the population estimate was calculated from the deduced parental alleles) to assess the likelihood of their occurring in a non-sister individual by chance. The probability of misclassification of drifters has been calculated by multiplying the frequency of the paternal allele with the mean frequency of the maternal alleles (estimated from the deduced colony parents). This estimation is carried out for each locus, and the results are multiplied to give the probability of a non-natal individual being misclassified by chance in a method similar to that used by Nanork et al. (2007) and Takahashi et al. (2010). The probability is as follows:

$$\prod_i P_{i(1)}\left(P_{i(2)}+P_{i(3)}\right),$$

where $P_{i(1)}$ is the frequency of the inferred father's allele at the ith locus, and $P_{i(2)}$ and $P_{i(3)}$ are the frequency of the inferred queen's first and second alleles, respectively, at the ith locus.

Males were identified either through direct morphological examination of adults and late stage pupae or because they were homozygous at all loci. For all but one colony (colony 10; Table 1) where only morphologically identifiable individuals were used, the inferred male parent had a different allele with those of the queen for at least one locus so that the diploid offspring could not be homozygous at all loci. We classified a male as a worker's son if he carried a paternal allele at one or more loci. It is possible that a worker-laid male only inherits maternal alleles, in which case he would not be detected as a worker's son. The probability of non-detection is 0.5^x where x is the number of informative loci (e.g. loci at which the male and queen carry different alleles).The total number of worker sons (detected and non-detected) was estimated by n / (1–0.5^x), where n is the number of worker sons that were detected and 1–0.5^x is the probability of detection. Drifter worker sons were so classified if they possessed an allele not corresponding to that of the natal bees. A drifter worker was considered a possible mother of a non-natal male found in the same colony if the male possessed an allele corresponding to that of the drifter worker at each locus.

Results

In total, 1,456 individuals from the 14 colonies were genotyped at the four loci (Table 2). Of these, 603 were identified as males. The majority of males were immature (eggs, larvae and pupae), 77 were adults and 9 had been dead for some time previous to collection (see additional material available online). The proportion of worker reproduction varied considerably between colonies (Table 1) and accounted for 78 of the 603 males detected (12.94 %). Worker reproduction was greater in colonies that were queenless (n=73 males in eight colonies), accounting for 12.11 % of overall males detected, while we found just five worker-produced males (0.83 %) amongst the six queenright colonies.

Ten non-sister (drifter) workers were found in five colonies (Table 1). They differed from their host bees at a mean of 4.7 alleles across the four loci. Four of these in colony 9 appeared to be a group of sisters and, notably, were also sisters to a female pupa within the same colony, apparently indicating that a foreign queen had entered the colony relatively recently and laid eggs. Similarly, a non-natal immature female was found in colony 10 as well as a male egg. The immature female and male may have been siblings, but the male was not an offspring of the drifter workers found in this colony (see supplementary material available online). Interestingly, the genotype of one of the drifter

Table 1 Summary of non-natal bees and brood and worker reproduction in 14 colonies of *B. terrestris*

				Genotyped				Foreign individuals					Natal-worker reproduction	
Reference number	Date collected (dd/mm/yy)	Total adults, eggs and brood	Queen	Adults	Eggs	Brood items	Total	Probability of misclassifying a non-natal	Adults (*n*)	Description	Offspring (*n*)	Description	Sons (*n*)	Description
1	8/8/09	24	Present	13	0	0	13	1.49×10^{-5}	0	–	0	–	0	–
2	10/8/09	352	Present	180	9	124	313	1.04×10^{-6}	0	–	0	–	0	–
3	13/8/09	145	Absent	57	29	19	105	2.10×10^{-5}	1	Queen	0	–	5	Eggs
4	14/8/09	313	Absent	37	83	101	221	1.96×10^{-6}	1	Worker	0	–	0	–
5	19/8/09	38	Absent	7	0	8	15	4.23×10^{-9}	0	–	0	–	0	–
6	19/8/09	159	Absent	31	21	18	70	6.30×10^{-4}	0	–	0	–	34	4 Eggs, 1 larva, 6 pupae, 1 adult
7	15/9/09	98	Present	14	37	35	86	4.01×10^{-6}	0	–	0	–	0	–
8	16/7/10	15	Absent	12	0	0	12	3.21×10^{-7}	0	–	0	–	0	–
9	29/7/10	44	Present	8	21	7	36	5.61×10^{-7}	4[a]	Workers	2	1 Pupa (female); 1 egg (male)	0	–
10	17/8/10	65	Absent	14	0	8	22	3.92×10^{-4}	3[b]	Workers	2	1 Egg (male); 1 pupa (female)	0	0
11	17/8/10	224	Absent	19	93	2	114	2.35×10^{-6}	1[c]	Worker	2	2 Pupae (male)	3	Eggs
12	2/9/10	676	Present	130	72	93	295	1.34×10^{-4}	0	–	0	–	1	Adult
13	3/9/10	96	Absent	14	39	3	56	1.25×10^{-5}	0	–	0	–	31	Eggs
14	7/9/10	103	Present	43	6	49	98	9.06×10^{-6}	1[d]	Worker	1	1 Pupa (male)	4	Eggs
Total	–	2,352	42.9 %	579	410	467	1456	8.59×10^{-5}	11	–	7	–	78	–

[a] Workers and female pupa appeared to be sisters. The egg was either a brother or son of these workers

[b] One of the drifter workers' genotype was consistent with that of the workers from colony 11. The "foreign offspring" may be siblings of each other but were not related to the drifter adults

[c] Pupae may be brothers, but the drifter worker was discounted as their mother

[d] Foreign worker did not lay the pupa

Table 2 Allelic richness of four microsatellites in *B. terrestris*, calculated using inferred genotypes of colony queens

Locus	Number (N)	Alleles	H_{Expected}	H_{Observed}
B126	14	10	0.88	0.93
B10	14	12	0.92	0.93
BT09	14	6	0.74	0.50
BT26	14	14	0.93	0.86
Mean	14	10.5	0.87	0.80

workers found in colony 10 was consistent with that of bees from colony 11. It should be noted that the entrances to these two colonies were close (approximately 2.5 m apart). A further colony (3) contained a foreign, worn queen.

A total of four male offspring of drifters were detected in three colonies (Table 1). The mean natal-worker reproduction across 13 colonies (n=476 natal workers) was 0.164 males per natal worker, whereas drifters (n=6) produced an average of 0.667 sons each. Colony 9 was removed from the calculation as the non-natal bees appeared not to be drifters. If we consider only the four colonies where drifting had taken place (following Takahashi et al. (2010)), then natal-worker reproduction amounts to just 0.092 sons per bee.

Discussion

Our data add to a growing body of literature which demonstrates that the reproductive strategies of bumblebees are more flexible than was once thought. In 14 wild bumblebee colonies, we find evidence for reproduction by workers, drifting of workers between colonies, reproduction of drifters within colonies and possible egg dumping by queens from foreign colonies.

Natal-worker reproduction in bumblebees varies considerably between species. For example, in a sample of five *Bombus wilmattae* colonies, workers produced the majority of males (Huth-Schwarz et al. 2011). However, in 32 *B. terrestris* colonies, natal workers produced just 2.2 % of male offspring. The presence or absence of the queen may have a profound effect on worker reproduction in many social hymenopterans (Beekman and Oldroyd 2008) as has been shown in *A. cerana* (Nanork et al. 2007) as well as several *Bombus* species. Within eleven *B. deuteronymous* colonies, natal workers had produced males in six colonies (Takahashi et al. 2010). On average, 27 % of males in six queenless colonies were worker males versus 16 % of males in the five queenright colonies. This pattern was also found in *Bombus ignites* where workers produced 5 and 57 % of males in queenright and queenless colonies, respectively (Takahashi et al. 2008). In ten *B. terrestris* colonies, Alaux et al. (2004) found a greater number of worker-derived males in colonies in which the queen had recently died (within 20 days, taken to be less than the male development time). In our queenright colonies, only 0.83 % of males appeared to have been workers' sons, whereas this percentage was 12.11 % in queenless colonies.

Lopez-Vaamonde et al. (2004) showed that drifting could occur between workers of laboratory-reared colonies which were allowed to forage outside, and it is clear that drifting may occur frequently when many bee colonies are artificially contained in close proximity to one another (Birmingham et al. 2004; Birmingham and Winston 2004; Lefebvre and Pierre 2007). Lower rates of drifting have also been found, comparable to those described here, in wild colonies of *B. deuteronymous* (Takahashi et al. 2010). Four *B. deuteronymous* drifters were reported in three of the eleven colonies that were examined. Discounting colony 9 (as it appears a queen was responsible for the non-natal bees here), 4 of 13 colonies contained drifters and drifter offspring. Takahashi et al. (2010) suggest that *B. deuteronymous* may be vulnerable to social parasitism because it is a surface-nesting species, and therefore, colonies may be easy to locate. However, *B. terrestris* nest in subterranean tunnels, which may extend for several metres (up to 6 m long; SO personal observation), appears to suffer from drifting at a similar frequency. What determines whether workers become drifters remains unclear; it seems likely that some or perhaps all drifters are workers from failed colonies, but it would require exhaustive monitoring of a large number of colonies in a particular area to test whether this is so.

In studies using laboratory-reared colonies, drifter reproduction was greater than resident-worker reproduction. For example, in queenless *B. terrestris* colonies, drifter workers produced 28.1 % of males, whereas resident workers were responsible for 19.0 % of males (Lopez-Vaamonde et al. 2004). The same study also showed that in queenright colonies sampled before the competition point (the date the first worker egg is laid (Duchateau and Velthuis 1989)), drifter reproduction accounted for significantly more males than resident workers (17 and 1, respectively). In *B. deuteronymous* colonies, Takahashi et al. (2010) found drifter workers produced 20 times more offspring per individual than resident workers in colonies where drifting occurred. In line with these findings, we found that drifter reproduction was approximately seven times greater than that of natal-worker bees. Not all of the eggs laid by natal or non-natal workers will develop into adults due to worker and queen policing of eggs (Duchateau 1989; Beekman and Oldroyd 2008). Extensive worker policing has been found in colonies of *A. cerana* (Oldroyd et al. 2001) and in *B. terrestris* colonies; the majority of worker-laid eggs are destroyed within 2 h of being laid (Zanette et al. 2012). In this study, nine of the worker-produced males detected had developed beyond the egg (and therefore policing) stage.

Perhaps the most unexpected of our results is the finding in two colonies of female pupae which were not laid by the resident queen but which appear to be sisters to adult workers within the colony. A foreign queen was found in a third colony. Usurpation of colonies by queens has been documented in bumblebees (Sladen 1912; Alford 1975; Paxton et al. 2001; Carvell et al. 2008). For example, Paxton et al. (2001) found several bees of similar lineage within a single colony of *B. hypnorum*, and Carvell et al. (2008) found that 30 *B. terrestris* queens had invaded 18 of 48 experimental colonies housed in boxes. However, colony usurpation is typically thought to occur primarily in spring, before the emergence of the second brood of workers, and is carried out by late-emerging queens (Alford 1975; Goulson 2010). Given that our colonies were sampled in July, August and September, this appears to be more akin to egg dumping, whereby queens enter a colony, lay a batch of eggs and then leave. This behaviour has been recorded in invertebrates such as lace bugs (*Gargaphia solani*) (Tallamy 2005), lady bird beetles (*Adalia* species) (Tallamy 2005; Ferrer et al. 2011) and in the ant *Leptothorax tuberum* (Roberts et al. 1999), but this was not previously known from bumblebees. Given the lateness of these events, it is unclear whether the queens engaging in egg dumping are old queens from the previous year or young queens produced in the current year. At least in the colonies we studied, their effort would seem to be ineffective as a reproductive strategy since they resulted only in worker offspring, but it seems likely that this may sometimes result in new queens being reared. It is also possible that non-natal male offspring may have been laid by queens rather than drifting worker bees.

Movement of bees between colonies has potentially important consequences for disease transmission. It has been assumed that the primary route of horizontal parasite transmission between bumblebee colonies is through infected bees visiting flowers which are later visited by uninfected individuals (Imhoof and Schmid-Hempel 1999). Infections of the protozoa gut parasite *Crithidia bombi* have been shown to spread rapidly between individuals within colonies which are thought to be due to the close proximity of individuals, use of shared nectar pots and lack of destructive UV light (Rutrecht et al. 2007). Thus, if drifters were carriers of a transmittable parasite, colony mates might be infected rapidly. *Nosema bombi* has been found to severely reduce or entirely prevent the production of new reproductives by colonies (Otti and Schmid-Hempel 2008), and so, it must rely upon horizontal transmission between colonies in order to infect future generations. Seasonal patterns of disease prevalence in sympatric bumblebee species can be markedly different even when they share many of the same floral resources (Goulson et al. 2012), suggesting that mechanisms such as worker drift which result in intraspecific transmission may be more important.

Drifting and egg dumping also have implications for bumblebee conservation for they have the potential to increase the effective population size. Monogamous social insects such as *B. terrestris* are at enhanced risk of inbreeding since the large majority of the population produce no offspring (Goulson et al. 2008; Whitehorn et al. 2009). Successful drifter reproduction, or reproduction by multiple queens in a single colony, would increase the effective population size and so reduces rates of genetic drift in small populations.

Genetic techniques provide a fascinating window on the colony structure of social insects. Further insights, for example, into the origins of drifters and egg-dumping queens, could be obtained by intensive sampling of colonies within a defined area and over multiple years.

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