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Research article

Non-lethal sampling of DNA from bumble bees for conservation genetics

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Summary. Non-lethal sampling of DNA from individuals in wild populations will often be required for studies of the conservation genetics of social insects, since it avoids destroying members of scarce or declining species. We investigated the effectiveness and consequences of methods of non-lethal sampling of DNA from bumble bee workers. In an experiment with two captive and confined *Bombus terrestris* colonies, we found that, unlike sampling haemolymph, sampling the terminal portion of the tarsus of a mid-leg of a worker reliably yielded amplifiable microsatellite DNA and did not significantly reduce worker survivorship. In a further experiment with four *B. terrestris* colonies whose workers were allowed to forage freely at flowers in the external environment, tarsal sampling of either a mid-leg or a hind-leg had no significant effects on worker survivorship, the mean body mass of foraging workers, the frequency or duration of foraging trips, mass of pollen loads or mass of nectar loads. We therefore suggest that tarsal sampling of either a mid-leg or a hind-leg is an effective and acceptable means of non-lethally sampling DNA from workers in wild populations of bumble bees, because effects on individual and colony performance are likely to be absent or minimal.

Key words: *Bombus*, conservation biology, microsatellite, non-destructive sampling.

Introduction

The study of the population genetics of wild populations of scarce and declining species can make an important contribution to their conservation management (Frankham et al., 2002; Smith and Wayne, 1996). The conservation genetics of social insects has, however, barely been explored. This is despite its involving several unique aspects (Chapman and Bourke, 2001; Pamilo and Crozier, 1997) and despite the very large number of studies on the evolutionary genetics of social insects (Pamilo et al., 1997; Ross, 2001). To advance the practical study of the conservation genetics of social insects, it is important to identify techniques for the non-lethal sampling of genetic material from free-living individuals, since in many cases it will be undesirable to destroy large numbers of individuals from small or declining populations (cf. Gerken et al., 1998; Rose et al., 1994). This will be particularly true in social insect species with small colony sizes, since the loss of sterile workers would then have a relatively large effect on colony performance (Schmid-Hempel et al., 1993). Correspondingly, in species with large colony sizes, the removal of sterile workers from colonies will be less likely to be damaging. Non-lethal sampling of DNA from insects, including social insects, also has advantageous applications in behavioural studies (Fincke and Hadrys, 2001; Kurtz and Sauer, 1999; Starks and Peters, 2002; Watts et al., 2001).

Few previous studies have quantitatively assessed methods of non-lethal DNA sampling from insects for either conservation or behavioural investigations. Gerken et al. (1998) and Kurtz and Sauer (1999) extracted DNA from haemolymph non-lethally withdrawn from larval and adult scorpionflies, but they did not report survivorships of sampled and non-sampled adults. Rose et al. (1994) and Lushai et al. (2000) non-lethally sampled DNA from endangered butterflies by removing portions of wing, but they also did not report the survivorships of sampled and non-sampled individuals. Fincke and Hadrys (2001) found that, in a tropical damselfly, removing a tibia from adult males for DNA analysis did not significantly alter the length of time over which males were subsequently observed in the field. Starks and Peters (2002), in the only such study to date involving social insects, established that tibial samples non-lethally removed from *Polistes* wasps yielded a high rate of microsatellite DNA amplification. In addition, they found that sampled wasps appeared to perform the same tasks as non-sampled wasps, although they were present on nests at a

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significantly lower rate (20% less frequently) than non-sampled wasps over a 2 h period three days after sampling. The studies of Fincke and Hadrys (2001) and Starks and Peters (2002) establish removal of part of a leg (tibia) as an effective means of DNA sampling and suggest that the impact on sampled individuals' survivorship is absent or small. However, in both studies the monitoring of the effects of sampling was limited. Moreover, their findings cannot be automatically applied to those social insects (corbiculate bees) that employ their legs as specialist foraging tools.

Bumble bees (*Bombus*: Hymenoptera, Apidae) are a group in which many species have recently undergone severe declines in some regions (e.g. British Isles: Benton, 2000; Prys-Jones and Corbet, 1991). Bumble bee species are also prone to occurring as distinct populations or subspecies on islands (Estoup et al., 1996; Widmer et al., 1998), whose population sizes will necessarily be relatively small. The conservation genetics of this genus are therefore of particular interest and concern. In this study, we investigated methods for non-lethal sampling of DNA from bumble bees and the effects of sampling on worker survivorship and foraging performance. First, we investigated using captive, confined bumble bee colonies two potential methods of DNA sampling – extraction from sampled haemolymph (Gerken et al., 1998; Kurtz and Sauer, 1999) or from a severed tarsal tip (Fincke and Hadrys, 2001; Starks and Peters, 2002). This experiment established that tarsal sampling both yielded amplifiable DNA at a higher rate than haemolymph sampling and, unlike haemolymph sampling, did not reduce worker survivorship. As is well known, bumble bee workers gather pollen using a specialized 'pollen basket' on the tibia of each hind-leg (Proctor et al., 1996). We therefore next tested whether tarsal sampling (from either a mid-leg or a hind-leg) influenced the survivorship and foraging efficiency of unconfined workers foraging freely at flowers in the external environment. Our results show that tarsal sampling represents an effective and benign method of non-lethally sampling DNA from bumble bees in the field, and is therefore likely to be a valuable technique in their conservation genetics.

Materials and methods

General

The study species was the common Palaearctic bumble bee species *Bombus terrestris*. Six queenright *B. terrestris* colonies (i.e. containing the colony queen) were obtained from a commercial supplier (Koppert U.K. Ltd, Homefield Road, Haverhill, Suffolk, CB9 8QP). All work was conducted at the Institute of Zoology, Regent's Park, London, between November and December 2001 (Experiment 1) and July and September 2002 (Experiments 2a and 2b).

Experiment 1 (Colonies 1 and 2)

DNA sampling: We tested whether haemolymph sampling (extraction of haemolymph) or tarsal sampling (removal of the terminal tarsal segments of a leg) represented the most suitable method for non-lethal DNA sampling from *B. terrestris*. For both methods, we compared the survivorship of sampled workers with that of a non-sampled control group of nestmate workers. We also measured the effectiveness of each sampling method in terms of the success rate of DNA amplification.

The experiment was carried out using Colonies 1 and 2, which were obtained from Koppert U.K. Ltd on 31 October and 14 November respectively and housed in a climate-controlled room. The colonies were transferred to artificial, wooden nest-boxes (internal dimensions, 17.5 cm × 27.5 cm × 16 cm deep) and placed singly on a table in a flight room (3.2 m × 3.4 m × 2.4 m high), which was kept at 24–28 °C under a daily light regime of 14h of artificial daylight and 10 h of darkness. Workers were able to leave the nest-box from an exit hole and fly freely in the room, where liquid bee food ('Beehappy' supplemented sugar water; Koppert U.K.) was supplied *ad libitum* at feeders placed approximately 2 m from the nest entrance. The colonies were also fed with defrosted pollen (Koppert Biological Systems, Berkel en Rodenrijs, The Netherlands) placed in the nest-box every two days.

After 3–4 weeks, we randomly selected 50–75% of workers from a colony, placing each worker in a plastic (Universal) tube and chilling it in a cool box containing domestic 'blue ice bricks' for approximately 5 min. Once workers were anaesthetized, they were individually marked with numbered disks glued to the thorax. Approximately half of the chilled workers per colony were then randomly selected to be immediately sampled for DNA by either collecting haemolymph (Colony 1) or tarsal sampling (Colony 2) (Table 1). Small volumes (1–2 µl) of haemolymph were taken by puncturing the thorax using a glass microcapillary whose tip had been drawn into a pointed tube in a flame (Moret and Schmid-Hempel, 2000). We sampled haemolymph from the thorax

Table 1. Sample sizes (numbers of treatment and control workers) in the experimental colonies.

Colony	Date[a]	Treatment	N treatment workers	N control workers
1	26–27 November 2001	Haemolymph sampling	40[b]	39[b]
2	10–11 December 2001	Tarsal sampling (mid-leg)	29	30
A	8–9 July 2002	Tarsal sampling (mid-leg)	49	49
	15 July 2002	Tarsal sampling (mid-leg)	6	5
	29 July–2 August 2002	Tarsal sampling (mid-leg)	18	18
B	8–9 July 2002	Tarsal sampling (mid-leg)	48	49
	26 July–1 August 2002	Tarsal sampling (mid-leg)	25	24
C	6–7 August 2002	Tarsal sampling (hind-leg)	38	34
	28 August–3 September 2002	Tarsal sampling (hind-leg)	11	14
D	6–7 August 2002	Tarsal sampling (hind-leg)	30	31
	27 August–4 September 2002	Tarsal sampling (hind-leg)	22	22

[a] Entries show date of initial marking (of both treatment and control workers) and treatment followed by (for Colonies A–D) subsequent dates on which additional workers (incoming foragers) were marked and (in approximately half of the workers) treated. This additional marking and treatment was carried out to maintain the numbers of marked, treated foragers (see text).

[b] In Colony 1, nine workers lost their disks (3 treatment workers, 6 control workers), reducing the sample size to 37 treatment and 33 control workers.

Table 2. Survivorship of *B. terrestris* workers as a function of tarsal sampling (treatment). 'Cut': tarsal-sampled workers (mid-leg sampled in Colonies A and B, hind-leg sampled in Colonies C and D); 'Uncut': workers not tarsal-sampled. Within colonies, survivorships of cut and uncut workers were compared with two-tailed Mann-Whitney U-tests.

Colony	Treatment	Median survivorship (days)	Interquartile range	N workers	Mann-Whitney U-tests
A	Cut	6	0–11	72	W = 4926, p = 0.29
	Uncut	9	1–14	71	
B	Cut	6	0–10	72	W = 5023.5, p = 0.23
	Uncut	8	1–10	75	
C	Cut	16	6–21	49	W = 2541, p = 0.31
	Uncut	10	0–23	48	
D	Cut	7	1–13	52	W = 2977, p = 0.15
	Uncut	5	0–12	53	

rather than from the abdomen to avoid inadvertently sampling the worker's crop contents. The terminal part of the tarsus (approximately 2 mm in length, comprising the tarsus but excluding the metatarsus) of a single mid-leg (arbitrarily right or left) was removed using a clean scalpel. Non-sampled (control) workers were handled in the same way as sampled workers except that no samples was taken. In subsequent text, tarsal-sampled workers are referred to as 'cut' workers and the corresponding control workers as 'uncut' workers. All workers were returned to plastic tubes and allowed to warm to room temperature for about 10 min. before being replaced in their colony. For each colony, all marking and DNA-sampling was carried out over 1–2 days. Over the following 13–16 days, we measured worker survivorship by recording the identity of all marked individuals that were found dead. Experiments were done sequentially so that there was only a single experimental colony in the flight room at any one time. Both Colonies 1 and 2 remained queenright throughout the experiment.

Molecular methods

Sampled haemolymph was mixed immediately with 30 µl of 1 × TE buffer (Tris-HCl 10 mM, 1 mM EDTA, pH 8.0) and 3 µl of proteinase K (20 mg/ml) and incubated at 55 °C for 30 min. Following digestion, we heated the mixture to 100 °C for 10 min. to denature the proteinase K. Tarsal samples were homogenized using a microcentrifuge tube pestle in 50 µl of 1 × TE buffer. This mixture was digested following a similar protocol to that used for the haemolymph samples except that we used 5 µl of proteinase K.

To check the effectiveness of the two types of DNA sampling, we PCR-amplified the same microsatellite locus (B96: Estoup et al., 1995, 1996) for all sampled workers under the same conditions. In a reaction volume of 20 µl we used 5 µl of template DNA, 2.5 mM $MgCl_2$, 250 nM of each primer (with the forward primer fluorescently labelled), 300 nM of each dNTP and 0.5 units of Taq polymerase (Gibco). All reactions were initially denatured at 95 °C for 5 min., followed by 16 cycles at 95 °C for 30 s, 61–45 °C for 30 s (the temperature being reduced by 1 °C per cycle) and 72 °C for 30 s. This was followed by 29 cycles at 95 °C for 30 s, 45 °C for 30 s and 72 °C for 30 s and a final extension of 7 min. at 72 °C. Amplified PCR products were sized on an ABI PRISM 373 automated sequencer with reference to an internal size marker, TAMRA 500 (Applied Biosystems).

Experiment 2a (Colonies A and B)

Sampling and conditions

Because the results of Experiment 1 showed that, unlike haemolymph sampling, tarsal sampling did not reduce worker survivorship and always yielded amplifiable DNA (see 'Results'), we conducted a further set of experiments. In Experiment 2a, we tested whether tarsal sampling using a mid-leg influenced the foraging efficiency of workers allowed to forage freely at flowers in the external environment, given that workers from Colonies 1 and 2 could forage only in a flight-room at artificial feeders.

Experiment 2a was carried out using Colonies A and B, which were obtained from Koppert U.K. Ltd on 4 July. After 4–5 days, all workers in each colony were individually marked with numbered disks glued to the thorax following anaesthesia by chilling, as described for Colonies 1 and 2. Immediately after marking, tarsal samples from a mid-leg were taken from a random selection of half of the workers, following the procedure used in Colony 2 (Table 1).

Colonies were housed throughout the experiment in the nest-box provided by the supplier (a plastic and polystyrene box with internal dimensions, 24 cm × 21 cm × 11 cm deep), which was placed inside a climate-controlled room (25–29 °C, 60% RH) lit by red light. The nest-box was connected by a 15cm-long tube of fine wire mesh to a wooden ante-chamber with internal dimensions of 17.5 cm × 27.5 cm × 16 cm deep and with a removable, clear Perspex lid. This in turn was connected by a tube of corrugated black plastic (150 cm long × 3 cm diameter) to the exterior, to which the tube led via a hole in the wall of the room. By leaving via the tube, workers were therefore able to forage freely outside (at wild and cultivated flowers in surrounding parkland and gardens), but when both leaving the nest and returning to it they had to pass through the ante-chamber. The colonies were allowed access to outside forage from the evening of 9 July, when the supplier's reservoir of 'Beehappy' liquid bee food, on which the colonies had subsisted, was removed. A visual census of all marked workers found in the colony was carried out every 2–3 days to estimate survivorship. Marked workers whose presence was recorded during the measurements of foraging efficiency (see below) were also included in the censuses, by adding them to the most recent census. Because the supply of marked foragers decreased due to forager mortality, additional workers were marked during the experiments in Colonies A–D (and, in the case of approximately half the workers, treated by tarsal sampling) (Table 1). All such additionally marked workers were incoming foragers, in order that foragers (which formed a subset of all workers) might be preferentially marked. The queens of Colonies A and B were found dead from unknown causes on 30 July and 16 July, respectively. In these and other cases of queen death, because the queenless workers continued to forage, we carried on using the colonies for data collection. On the evening of 2 August, following the end of all observations, the nest-boxes housing Colonies A and B were adjusted using the supplier's entrance valve such that workers still outside could return but none could leave. Four days later both colonies were frozen and a final census of all remaining workers was conducted. Note that Colonies A–D were all terminated before the eclosion of any sexual pupae. This meant that no reproductives of these commercially-reared bees, which derive from populations other than U.K. *B. terrestris*, were released into the external environment.

Pollen loads

Between 15 and 22 July, Colonies A and B were observed on alternate days to measure pollen loads (3 days per colony). Each colony was

observed approximately continuously from around 0930 to 1630, except for breaks between roughly 1030 and 1045, 1230 and 1300, and 1530 and 1545. The time of departure was recorded for each worker leaving the ante-chamber and entering the tube to the exterior. On the worker's return, the time was again recorded and the worker was removed from the ante-chamber with forceps. All pollen in the pollen baskets of both hind-legs was gently removed using an entomological pin and weighed on an electronic balance (Oxford S1204) to the nearest 0.0001 g. Measurements of pollen mass were highly repeatable, with the mean coefficient of variation from the same pollen masses of ten foragers each weighed ten times in succession being 0.8% (range 0.2–3.1%). The unladen worker was then weighed. All workers were replaced in the ante-chamber after weighing, as was any removed pollen (which was then soon discovered by the original forager or by other workers and consumed).

Nectar loads

Between 24 July and 2 August, Colonies A and B were observed on alternate days to record nectar loads (4 days per colony). Colonies were observed as for the pollen load measurements. However, because foragers returning with nectar alone could not be externally identified as foragers, we adapted the procedure by weighing all workers that were on the point of leaving for the exterior. The workers were removed from the ante-chamber, weighed, and replaced in the ante-chamber, whereupon most departed to forage. Returning workers were weighed as before, with any pollen in the pollen baskets being removed, weighed and used for the pollen load analysis. The difference in mass between a returning forager (minus any pollen) and the same forager at the time of its departure was taken to be the mass of nectar collected (cf. Spaethe and Weidenmüller, 2002).

Experiment 2b (Colonies C and D)

Sampling and conditions

We repeated Experiment 2a using a hind-leg for tarsal sampling. Bumble bees employ their mid-legs to transfer pollen to the pollen basket situated on the tibia of the hind-leg (Proctor et al., 1996). Therefore we tested for any difference between the effects of tarsal sampling of a mid-leg and the effects of tarsal sampling of a hind-leg.

Experiment 2b was carried out using Colonies C and D, which were obtained from Koppert U.K. Ltd on 6 August. On the day of delivery, or one day later, all workers in each colony were individually marked as described for Colonies A and B. Immediately after marking, half the workers were randomly selected for tarsal sampling. This was carried out as for Colonies A and B, except that sampling was from a hind-leg and not from a mid-leg (Table 1). Because only the terminal part of the tarsus was sampled, the pollen basket, which is on the tibia of the hind-leg (and hence proximal to the metatarsus), was left intact. Regular visual censuses of the colonies were carried out as for Colonies A and B. The queens of Colonies C and D were found dead from unknown causes on 27 August and 2 September, respectively. The final census of Colonies C and D was carried out on 9 September, following closure of the colonies on 6 September (as in Colonies A and B, allowing foragers to return but not to leave).

Pollen loads

Between 9 August and 23 August, Colonies C and D were observed on alternate days to measure pollen loads (5 days observation on Colony C and 4.5 days on Colony D). Methods were as for Colonies A and B, except that the pollen loads on the left and right hind-legs were weighed separately. This was to investigate whether the sidedness of the hind-leg that was cut (i.e. whether it was the left or right leg) affected the symmetry of the pollen load.

Nectar loads

Between 27 August and 6 September, Colonies C and D were observed on alternate days to measure nectar loads (4 days observation on Colony C and 5 days on Colony D). Methods were as for Colonies A and B.

Statistical analyses

In Experiment 2, almost all datasets of continuous data (body mass, foraging trip duration, pollen load, nectar load) were normally distributed (4 of 35 datasets significantly non-normally distributed: Kolmogorov-Smirnov tests) and so were analysed with parametric statistics. The survivorship and foraging trip frequency datasets were not normally distributed and were analysed with non-parametric statistics. The effect of the treatment (tarsal sampling) on foraging trip duration, pollen load and nectar load was analysed using General Linear Models designed as mixed model two-way ANOVAs, with colony as a random factor and treatment as a fixed factor. Within treated workers, the effect of sampling a mid-leg or a hind-leg on foraging trip duration, pollen load and nectar load was analysed using General Linear Models designed as mixed model one-way nested ANOVAs, with leg-type as a fixed factor and colony as a nested, random factor. We addressed the power of our tests by calculating the 95% confidence intervals of the differences between average values for treatment (cut) and control (uncut) workers, expressed as a percentage of the average value for the control workers. Smaller values of these confidence intervals, and a higher degree of symmetry about 0%, imply greater confidence in the null hypothesis of no difference between the treatments and controls. In the body mass, foraging trip duration, pollen load and nectar load datasets, some individual workers contributed more than one data point, since individual workers made more than one foraging trip in the course of the experiment. To avoid pseudoreplication in the data, the values used in the analyses were therefore the mean values per worker over all its foraging trips.

Results

Experiment 1

Worker survivorship

In Colony 1 (with haemolymph-sampled workers), a significantly higher fraction of sampled workers (initial N = 37) than of control workers (initial N = 33) had died by the end of the experiment (60% v. 9%; $\chi^2 = 16.11$, d.f. = 1, $p < 0.0001$). By contrast, in Colony 2 (with tarsal-sampled workers), there was no significant difference between the fractions of sampled workers (initial N = 29) and of control workers (initial N = 30) that died by the end of the experiment (17% v. 13%; $\chi^2 = 0.17$, d.f. = 1, $p = 0.68$). Therefore, haemolymph sampling significantly increased worker mortality whereas tarsal sampling did not.

Microsatellite amplification

The success rate of amplifying microsatellites (measured as the presence or absence of scorable alleles on the chromatogram) from haemolymph DNA was significantly lower (15% of 40 samples) than the corresponding success rate for DNA derived from tarsal samples (100% of 29 samples) ($\chi^2 = 48.60$, d.f. = 1, $p < 0.0001$). Therefore, DNA was much more effectively extracted and amplified from tarsi than from haemolymph.

Experiments 2a and 2b

Worker survivorship

In Experiment 2, survivorship could not be measured in terms of the number of workers of each class remaining at the end of the experiment as in Experiment 1. This was because

Table 3. Body mass of foraging *B. terrestris* workers as a function of tarsal sampling. 'Cut' and 'Uncut' are defined as in Table 2. SE: standard error. Within colonies, body masses of cut and uncut workers were compared with two-tailed t-tests.

Colony	Treatment	Mean body mass (g)	SE	N workers	t-tests
A	Cut	0.184	0.013	18	t = 0.46, d.f. = 47, p = 0.65
	Uncut	0.190	0.008	31	
B	Cut	0.216	0.009	30	t = 0.84, d.f. = 65, p = 0.40
	Uncut	0.228	0.010	37	
C	Cut	0.183	0.007	24	t = 0.92, d.f. = 47, p = 0.36
	Uncut	0.193	0.009	25	
D	Cut	0.212	0.009	34	t = 0.48, d.f. = 64, p = 0.63
	Uncut	0.218	0.009	32	

most marked workers disappeared during the course of the experiment, which suggests that they died outside the nest while in the external environment. Survivorship was therefore measured as the length of time in days between the marking of a worker and the last record of it during the censuses. This measure may have incorrectly estimated true survivorship, because, for example, workers may have been overlooked during the censuses. (In addition, the ages of the workers when marked were unknown.) However, since individual colonies initially contained equal numbers of marked cut and uncut workers, the measure was not biased with respect to the tarsal sampling treatment. Median survivorship varied between colonies, with Colony C workers having particularly high survivorship, but within colonies there were no significant differences between cut and uncut workers (Table 2). The 95% confidence intervals for the differences between the medians were −33%–0% (Colony A), −38%–0% (Colony B), −20%–70% (Colony C) and 0%–100% (Colony D). This suggested a trend for tarsal sampling of a mid-leg to reduce survivorship and for tarsal sampling of a hind-leg to increase it (Table 2). However, it is more likely this was a chance outcome, because it is difficult to imagine a mechanism by which tarsal sampling might have opposite effects on survivorship depending on the leg cut.

Body mass

Not all the workers marked at the start of the experiments foraged, so it was possible that tarsal sampling influenced the mean body mass of the subset of workers that became foragers. For workers that were marked as known foragers (Table 1), tarsal sampling could have influenced whether they remained as foragers as a function of their body mass. We tested for such effects using the measures of body mass of returning foragers from both the pollen-load and nectar-load observation periods. These measures excluded the mass of any pollen carried but included the mass of any nectar carried, since in the collection of data on pollen loads workers were not weighed before their departure and therefore bore nectar loads of unknown size. There were no significant differences between the body masses of cut and uncut foraging workers within colonies (Table 3). The 95% confidence intervals for the differences between the means were −18%–12% (Colony A), −17%–7% (Colony B), −18%–7% (Colony C) and −15%–9% (Colony D).

Table 4. Foraging trip frequency of *B. terrestris* workers as a function of tarsal sampling. Data are for those foraging trips for which the start and end of the trip were observed (Table 5). 'Cut' and 'Uncut' are defined as in Table 2. Within colonies, foraging trip frequencies of cut and uncut workers were compared with χ^2 tests (all d.f. = 1, all p > 0.5).

Colony	Treatment	Total N foraging trips	χ^2	Total N workers making foraging trips	χ^2
A	Cut	15	0.04	9	0.06
	Uncut	13		9	
B	Cut	16	0.03	12	0.00
	Uncut	16		13	
C	Cut	15	0.26	13	0.04
	Uncut	19		13	
D	Cut	27	0.42	15	0.00
	Uncut	33		16	

Foraging trip frequency and duration

There were no significant differences between either the total number of foraging trips made by cut and uncut workers within colonies, or the total number of workers making foraging trips (Table 4). Our measure of foraging trip duration may have incorrectly estimated the true duration of foraging trips because, given observations were not continuous (see 'Materials and Methods'), not all departures and returns may have been recorded. However, since individual colonies initially contained equal numbers of marked cut and uncut workers, our measure was not biased with respect to the tarsal sampling treatment. In addition, the mean journey times recorded for uncut workers across all four colonies (mean ± SE = 52.6 ± 8.0 min; Table 5) resembled those recorded in previous studies (18–75 min.: Spaethe and Weidenmüller, 2002). There was no significant effect of tarsal sampling on foraging trip duration, and no significant effect of colony membership or significant interaction of colony and treatment (Table 5). The 95% confidence intervals for the differences between the means were −50%–68% (Colony A), −66%–75% (Colony B), −78%–38% (Colony C) and −34%–54% (Colony D). This analysis was relatively lacking in power because of the wide variation in foraging trip duration between individuals, but no consistent trend in the effect of tarsal sampling was evident (Table 5).

Table 5. Foraging trip duration of *B. terrestris* workers as a function of tarsal sampling. 'Cut' and 'Uncut' are defined as in Table 2.

Colony	Treatment	Mean foraging trip duration (min.)	SE	N workers
A	Cut	80.1	15.7	9
	Uncut	73.4	13.0	9
B	Cut	57.1	14.9	12
	Uncut	54.9	11.3	13
C	Cut	37.6	8.5	13
	Uncut	47.0	10.2	13
D	Cut	38.9	6.3	15
	Uncut	35.3	4.4	16

Two-way mixed model GLM results.

Effect	d.f.	SS	MS	F	p
Colonies A and B					
Colony	1	4532	4510	2.22	> 0.10
Treatment	1	176	203	3.98	> 0.25
Colony × Treatment	1	51	51	0.03	> 0.85
Error	39	79180	2030		
Colonies C and D					
Colony	1	389.3	380.6	0.50	> 0.45
Treatment	1	78.2	119.6	0.20	> 0.50
Colony × Treatment	1	596.2	596.2	0.78	> 0.35
Error	53	40375.1	761.8		

Table 6. Pollen load of *B. terrestris* workers as a function of tarsal sampling. 'Cut' and 'Uncut' are defined as in Table 2. Pollen loads are total pollen loads, i.e. summed over both hind-legs.

Colony	Treatment	Mean pollen load (g)	SE	N workers
A	Cut	0.011	0.002	11
	Uncut	0.020	0.003	14
B	Cut	0.017	0.003	17
	Uncut	0.017	0.002	18
C	Cut	0.007	0.002	7
	Uncut	0.005	0.001	4
D	Cut	0.013	0.003	19
	Uncut	0.020	0.004	13

Two-way mixed model GLM results.

Effect	d.f.	SS	MS	F	p
Colonies A and B					
Colony	1	0.000013	0.000032	0.26	> 0.60
Treatment	1	0.000208	0.000294	1.00	> 0.25
Colony × Treatment	1	0.000293	0.000293	2.33	> 0.10
Error	56	0.007030	0.000126		
Colonies C and D					
Colony	1	0.000779	0.000875	5.25	< 0.05
Treatment	1	0.000252	0.000054	0.34	> 0.50
Colony × Treatment	1	0.000159	0.000159	0.95	> 0.30
Error	39	0.006498	0.000167		

Comparing cut workers, we found a trend for workers sampled at a mid-leg to have greater foraging trip durations than those sampled at a hind-leg (mean ± SE = 66.9 ± 11.0 min. and 38.3 ± 5.1 min. respectively, N = 21 and 28 respectively), but this difference was not significant ($F_{1,2} = 7.99$, $p > 0.1$). Note, however, that all comparisons of mid-leg and hind-leg cutting are potentially confounded by time, since Colonies A and B were studied earlier in the season (15 July–2 August) than Colonies C and D (9 August–6 September). Therefore, cut workers from colonies A and B may have foraged for longer not because of the effect of cutting a mid-leg rather than a hind-leg, but, for example, because forage was more plentiful earlier in the season. Consistent with this, uncut workers from Colonies A and B also tended to have greater average foraging trip durations than those from Colonies C and D (mean ± SE = 62.5 ± 8.6 min. and 40.5 ± 5.2 min. respectively, N = 22 and 29 respectively; $F_{1,2} = 4.61$, $p > 0.25$).

Pollen load

There was no significant effect of tarsal sampling on the pollen loads returned by foraging workers (Table 6). Neither was there any significant interaction of colony and treatment, but in Experiment 2b there was a significant effect of colony, with Colony C workers having significantly smaller average pollen loads than Colony D workers (Table 6). The reasons for this difference were unknown, but could have stemmed from inherent differences in the foraging abilities of workers from the two colonies. The 95% confidence intervals for the differences between the means were −90%–0% (Colony A), −47%–47% (Colony B), −80%–160% (Colony C) and −90%–20% (Colony D). This analysis was relatively lacking in power because of comparatively wide between-individual variation and small sample sizes. In two colonies (A and D), there was a trend for cut workers to have smaller pollen loads (55–65% of those of uncut workers), but in the other two colonies (B and C) the mean pollen loads of cut and uncut workers were identical or very similar (Table 6). Comparing cut workers, we found no significant difference between the pollen loads brought back by workers sampled at a mid-leg and those sampled at a hind-leg (mean ± SE = 0.015 ± 0.002 g and 0.012 ± 0.002 g respectively, N = 28 and 26 respectively; $F_{1,2} = 0.87$, $p > 0.25$), though note again that this comparison was potentially confounded by the difference in observation dates (see above). The mean pollen load of uncut workers (N = 49) from all four colonies (0.018 g: Table 6) was of the same order as, though less than, the mean pollen load (0.025 g, N = 42) reported in native *B. terrestris* workers by Free (1955).

In uncut workers from Colony D, there was no significant difference between the mass of pollen carried on the left hind-leg and that carried on the right hind-leg (mean ± SE = 0.0102 ± 0.0020 g and 0.0107 ± 0.0021 g respectively; paired t-test, $t = 0.46$, d.f. = 12, $p > 0.65$). (This analysis was not carried out for uncut workers of Colony C, because of the small sample available in this colony: Table 6.) In cut workers from Colonies C and D, there was also no significant difference between the mass of pollen carried on the left hind-leg and that carried on the right hind-leg (Colony C: mean ± SE = 0.0031 ± 0.0007 g and 0.0039 ± 0.0012 g respectively; paired

t = 1.21, d.f. = 7, p > 0.25; Colony D: mean ± SE = 0.0070 ± 0.0016 g and 0.0066 ± 0.0017 g respectively; paired t = 1.11, d.f. = 19, p > 0.25). In addition, the mean size of the difference between the pollen masses borne on the right and left legs did not differ significantly between workers whose left hind-legs were cut and those whose right hind-legs were cut (left hind-leg cut: mean ± SE pollen mass = 0.0051 ± 0.0014 g for left leg and 0.0055 ± 0.0016 g for right leg; right hind-leg cut: mean ± SE pollen mass = 0.0066 ± 0.0019 g for left leg and 0.0061 ± 0.0020 g for right leg; t-test on differences, t = 1.48, d.f. = 24, p > 0.10, N = 13 left-cut workers and 15 right-cut workers pooled from Colonies C and D to increase the sample size). These findings indicated that uncut workers had no preference for carrying more pollen on one side over the other and that the sidedness of the hind-leg chosen to be cut had no effect on the sidedness of pollen storage across the two hind-legs.

Nectar load

Sample sizes for nectar load measures were relatively small because not all foragers could be weighed both before and after their foraging trips. There was no significant effect of tarsal sampling on the nectar loads returned by foraging workers and no significant effect of colony or significant interaction of colony and treatment (Table 7). The 95% confidence intervals for the differences between the means were −60%–81% (Colony A), −37%–37% (Colony C), and −30%–64% (Colony D). (No confidence interval is given for Colony B because in this colony the dataset included a single cut worker.) Again, this analysis was relatively lacking in power because of comparatively wide between-individual variation and small sample sizes. However, in this case the trend was for tarsal sampling to increase nectar load, with cut workers in two colonies (A and D) having non-significantly larger nectar loads than uncut workers (11–18% larger) (Table 7). Comparing cut workers, we found no significant difference between the nectar loads brought back by workers sampled at a mid-leg and those sampled at a hind-leg (mean ± SE = 0.042 ± 0.006 g and 0.048 ± 0.006 g respectively, N = 7 and 18 respectively; $F_{1,2}$ = 0.001, p > 0.75), though once more this comparison was potentially confounded by the difference in observation dates.

Table 7. Nectar load of *B. terrestris* workers as a function of tarsal sampling. 'Cut' and 'Uncut' are defined as in Table 2.

Colony	Treatment	Mean nectar load (g)	SE	N workers
A	Cut	0.041	0.007	6
	Uncut	0.037	0.009	5
B	Cut	0.049	–	1
	Uncut	0.052	0.012	4
C	Cut	0.038	0.005	5
	Uncut	0.038	0.004	11
D	Cut	0.052	0.008	13
	Uncut	0.044	0.007	12

Two-way mixed model GLM results

Effect	d.f.	SS	MS	F	p
Colonies A and B					
Colony	1	0.000530	0.000335	0.80	> 0.35
Treatment	1	0.000025	0.000001	0.02	> 0.75
Colony × Treatment	1	0.000036	0.000036	0.09	> 0.75
Error	12	0.005060	0.000422		
Colonies C and D					
Colony	1	0.001000	0.000877	1.97	> 0.15
Treatment	1	0.000225	0.000126	1.10	> 0.25
Colony × Treatment	1	0.000115	0.000115	0.26	> 0.60
Error	37	0.016434	0.000444		

Discussion

Methods for non-lethally sampling DNA from individuals in wild populations of social insects are required for studies of both conservation genetics and behaviour. We found that microsatellite DNA could be reliably amplified from a tarsal tip of a worker bumble bee but not from a haemolymph sample. Haemolymph sampling, but not tarsal sampling, significantly reduced the survivorship of confined workers relative to controls. In workers allowed to forage freely at flowers in the external environment, tarsal sampling had no significant effects on survivorship, body mass of foragers, the frequency or duration of foraging trips, mass of pollen collected or mass of nectar collected. We detected no significant differences between the effects of sampling a mid-leg and those of sampling a hind-leg, or (in the hind-leg trials) between sampling a left leg or a right leg.

Some of our analyses had relatively low power due to a combination of comparatively wide between-individual variation and limited sample sizes. Sample sizes were limited because, although many workers were marked and treated, only a subset acted as foragers and, of these, only a further subset yielded the requisite data on foraging trip duration, pollen load or nectar load. It is therefore possible that a more extensive study would have revealed significant effects of tarsal sampling. However, tarsal sampling clearly does not completely prevent workers from foraging. In addition, for the main traits investigated we found no consistent trends in the effects of tarsal sampling (worker survivorship, body mass, foraging trip duration), trends for negative effects (pollen load), and trends for positive effects (nectar load), suggesting a lack of consistent impairment by the treatment. Results from Colonies A and D suggested that tarsal-sampled workers might compensate for some reduction in their ability to collect pollen by increasing the amount of nectar foraging. Finally, in sampling in the field from workers caught outside the nest, e.g. at flowers, the number of workers sampled per colony is likely to be small. This means that any negative impacts from tarsal sampling on individual colonies, including any arising via behavioural traits not investigated in this study, are also likely to be small.

It is possible that employing more sophisticated methods of withdrawing haemolymph (e.g. through membranes between the abdominal tergites, since this would avoid any

damage to thoracic cuticle and muscles: Moret and Schmid-Hempel, 2000), or of extracting DNA from haemolymph (Gerken et al., 1998; Kurtz and Sauer, 1999), would have given different results to those of this study. However, we sought a non-lethal sampling method that was capable of easy implementation in the field and that involved rapid laboratory procedures. Tarsal sampling fulfils these criteria more completely than haemolymph sampling. In addition, loss of tarsal segments occurs due to natural wear in bumble bee workers (Cartar, 1992). This is also true of loss of wing tissue, so removing tissue from the wings represents another potential method of non-lethal DNA sampling in bumble bees. Studies in which *B. terrestris* workers were prevented from flying have shown that removal of around 50% of the area of each fore-wing is sufficient to prevent flying whereas removal of 10–20% of this area did not prevent flight or foraging (Doums and Schmid-Hempel, 2000; König and Schmid-Hempel, 1995). However, as reducing wing area most probably has a continuous effect on flight ability and overall performance, some impairment of these traits seems likely even if small amounts of wing are removed. This suggestion is supported by an experiment in which workers within *B. melanopygus* colonies were wing-clipped (Cartar, 1992). An average area of 2.1 mm^2 of each fore-wing was removed, amounting to an average reduction in fore-wing area of 25%. This level of wing-clipping significantly decreased the survivorship of free-foraging workers relative to controls. The duration of foraging bouts and the sizes of pollen loads were not affected, although the sizes of pollen loads were estimated and not measured by weighing. Nectar loads were not investigated (Cartar, 1992). Hedenström et al. (2001) found that clipping 10% of the area of each fore-wing from *B. terrestris* workers did not significantly increase the energetic cost of flight, but survivorship and proposed effects on manoeuvrability in flight were not investigated.

For these reasons, we recommend tarsal sampling of either a mid-leg or a hind-leg as an effective and benign method of non-lethally sampling DNA from individuals in wild populations of bumble bees. The technique is conceivably also applicable to non-flying social insects, such as dealate ant queens, worker ants, and worker termites. However, although dealate queens of the ant *Leptothorax acervorum* are apparently unimpaired by removal of tarsal segments (for marking) (Bourke, 1991, 1993, 1995), we know of no formal comparison of the behavioural effects of tarsal sampling in ants and termites. For implementation of tarsal sampling on bumble bee workers in the field, the equipment required would be Universal tubes for capturing and temporarily housing bees, cool box, scalpel, and Eppendorf tubes each containing 50 µl of 1×TE buffer for receipt of tarsal samples. The cool box would be both for anaesthesia of bees prior to sampling and for cold storage of the Eppendorfs containing buffer and tarsal samples. Alternatively, tarsal samples could be stored at ambient temperature in ethanol and later frozen for storage or used for DNA extraction.

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