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RESEARCH ARTICLE

A comparison of mark–release–recapture methods for estimating colony size in the wood ant *Formica lugubris*

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Abstract Colony size can be considered the analogue of the body size of a superorganism. Just as body size is important to the physiology of an individual animal, colony size correlates with the life-history and ecology of social insects. Although nest excavation and counting all individuals is the most accurate method for estimating colony size (or nest size), it has the major drawback of being destructive. Alternatively, mark–release–recapture (MRR) can be used repeatedly to measure the size of the same colony or nest. We compared the accuracy and feasibility of four MRR methods and a Mound-Volume method with complete counts from nest excavation for estimating the nest size of *F. lugubris*, a mound-building wood ant of the *Formica rufa* group, during the early spring in Scotland. We found that our After-Disturbing method, in which we performed marking and recapturing after gentle disturbance to the top of nest mound, has the best balance between accuracy, non-destructiveness, and time required. We also found that mound volume can be an index of ant nest size under certain conditions. Both non-destructive methods can be used on the same colony or nest repeatedly to monitor nest dynamics.

Keywords Red wood ants · Nest size · Mark–release–recapture method · *Formica rufa* group

Introduction

Body size is one of the most significant characteristics of an animal because it influences virtually all physiological characters (Blanckenhorn and Demont, 2004; Brown and Lomolino, 1998). In social insects, the colony can be considered the biological analogue of the "body" of an individual organism (Clémencet and Doums, 2007; Kaspari and Vargo, 1995; Lanan et al., 2011; Tschinkel, 1991, 1998, 1999). As a group of cells are categorised as an organism when the cells build a cooperative unit to reproduce their genes, it could be correct to classify a group of organisms as a superorganism when the organisms construct a cooperative unit to reproduce their genes (Seeley, 1989). Hence, the individual and the superorganism are two levels of organisation of social insects. Studying the "body size" of the colony may reveal how this life-history trait of the superorganism correlates with their lifestyle and habitat. For example, number of workers may be related directly to competitive abilities in ants (Palmer, 2004), foraging behaviour in ants and bees (Eckert et al., 1994; Herbers and Choiniere, 1996), and worker life span of wasps (O'Donnell and Jeanne, 1992; Pamilo et al., 1992). Moreover, it is especially interesting that the size of these superorganisms can have a wide range, for example, over eight orders of magnitude in the ant family Formicidae alone (Kaspari and Vargo, 1995). In addition, in polydomous ants, one colony may settle in either one nest or several spatially separated but socially connected nests (Hölldobler and Wilson, 1977). Although the nests and other structures of ant colonies can be regarded as extensions of the superorganism, Debout et al. (2007) suggested that nest-level allocation is subjected to stronger selection than is colony-level allocation in some polydomous ants. Banschbach and Herbers (1996) indicated that only nest-level traits play a major role in determining variation in fitness. Therefore, estimating colony size, as well as nest size of polydomous colonies, is important to understanding the life-history and ecology of social insects.

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The total number of individuals or the worker population in a colony (or a nest) has been used to represent the colony size (or nest size) of social insects (Kaspari and Vargo, 1995; Tschinkel, 1998, 1999). In ants, the most accurate colony size estimation method is nest excavation (Akre et al., 1994; Beshers and Traniello, 1994; Elmes, 1974; Gordon, 1992; Tschinkel, 1993; Tschinkel et al., 1995). Although the nest excavation method can obtain the exact count and biomass of all stages in the nest, it is destructive and laborious (Skórka et al., 2006; Stradling, 1970). An additional step has also been used to decrease labour of excavation method in some studies. After excavation and mixing the whole nest soil and ants, the number of individuals in a given sampled mound soil volume was counted, then the nest size was estimated by this number and the total mound volume (Tschinkel, 1993; Tschinkel et al., 1995).

Alternatively, mark–release–recapture (MRR) methods (or capture–mark–recapture, CMR) can monitor the colony dynamics without destroying it (Billick, 1999; Breen, 1979; Brown et al., 2002; Chew, 1959; Kruk-De Bruin et al., 1977; Rosset and Chapuisat, 2007; Sundström, 1995). In general, the MRR method is based on some assumptions (Chew, 1959; Stradling, 1970): (1) every individual in the colony is able to be captured and marked; (2) a sample which represents the population of the colony is taken to mark and estimate; (3) the marks are permanent during the sampling period, and the marked individuals are not influenced by them; (4) the marked animals mix thoroughly with unmarked individuals before resampling; (5) the population is closed, the rates of immigration and emigration are known and no births and deaths occur during the period of mixing. From these assumptions, probably the most challenging step of the MRR method is how to ensure all individuals are available to be captured and marked. For example, in carpenter ants, about 80 % of the individuals do not forage, so marking on foraging trails can only estimate the forager group (Ayre, 1962). It can also be a problem to capture certain special types of workers such as repletes which usually stay in the deep parts of the nests (Chew, 1959). Then there is a trade-off between the assumption (4) and the assumptions (3) and (5). To make sure marks are retained and the population is isolated needs a short experimental period. On the other hand, the period should be long enough for marked and unmarked ants to mix thoroughly. Several studies have investigated the feasibility of using non-destructive MRR methods to estimate real parameters of the colony, such as colony size and colony biomass (Table 1). Porter and Jorgensen (1980) and Kruk-de Bruin et al. (1977) showed that although the estimation from MRR method on foragers (on the trail) only represented the size of the foragers group, it can be used to estimate the whole colony size of *Pogonomyrmex owyheei* and *Formica polyctena* because foragers compose a certain proportion of colony. Besides the forager group, defender estimation was also a good index of colony size in *P. owyheei* (Porter and Jorgensen, 1980). Billick (1999) tested whether the MRR method provided an accurate estimate of worker number of *F. neorufibarbis* by capturing and marking workers after overturning rocks on the nests. Although the fit of the regression line showed by R^2 value from the studies of foragers is higher (Kruk-De Bruin et al., 1977; Porter and Jorgensen, 1980), the results underestimated the colony size. On the other hand, although the regression line in Billick's study has a lower fit compared to former studies, estimation from the number of workers rather than only of foragers seems to more realistically predict the colony size.

In addition to the above main methods, in some studies the colony size or nest size was related to or estimated by other non-destructive methods using the features of nest, such as the basal area or the volume of nest mound (Deslippe and Savolainen, 1994; Liautard et al., 2003; Savolainen et al., 1996; Sorvari, 2009; Sorvari and Hakkarainen, 2007). Table 1 shows several studies which compared the nest features with the real parameters of colony. Tschinkel (1993) and Tschinkel et al. (1995) suggest that mound volume of the nest may be a convenient and non-destructive method to estimate the colony biomass of *Solenopsis invicta*. Ground-level area of the mound was related to alate mass (Deslippe and Savolainen, 1994), and worker number (Savolainen et al., 1996). Surface area of the nest dome could predict both worker number and brood production (Liautard et al., 2003). Depth of the nest was also related to colony size (Clémencet and Doums, 2007). However, there are also problems with these methods of estimation. Domisch et al. (2008) argued that decomposition could be either increased or decreased by the activity of colony or external reasons such as temperature. Nests of *F. lugubris* and *F. polyctena* in shaded areas have higher mounds (Mabelis, 1979; Sudd et al., 1977). Breen (1979) also indicated that nest diameter was not a useful predictor for the forager population of *F. lugubris*.

Activities of workers or the colony was also measured to estimate colony size in some studies (Table 1). Skórka et al. (2006) conducted a new method for three *Myrmica* species in which they opened the topmost part of the nest until the first chambers with larvae was found and used sticks to remove workers which climbed up the stick. The number of workers removed in a given period was positively correlated with the number of workers in the nest. The amount of traffic on trails was combined with the number of trails radiating from a nest to estimate the relative size of a nest population of *F. polyctena* (Mabelis, 1979). A similar idea has been used to predict worker numbers of wasps by

Table 1 Summary of the results in studies which compared the estimates by non-destructive methods with real parameters of ant colony size

Estimates	Real parameters	R^2	*N*	Species	References
Mound volume	Colony biomass	0.90	30	*Solenopsis invicta*	Tschinkel et al. (1995)
Nest volume	Total number	0.87	16	*Formica pallidefulva*	Mikheyev and Tschinkel (2004)
Defender number (MRR)	Total adults	0.86	12	*Pogonomyrmex owyheei*	Porter and Jorgensen (1980)
Forager number (MRR)	Total number	0.86	15	*F. polyctena*	Kruk-de Bruin et al. (1977)
Mound volume	Colony biomass	0.85	75	*S. invicta*	Tschinkel (1993)
Removed worker number[a]	Worker number	0.83	21	*Myrmica ruginodis*	Skórka et al. (2006)
Forager number (MRR)	Total adults	0.81	10	*P. owyheei*	Porter and Jorgensen (1980)
Territory area	Worker biomass	0.80	30	*S. invicta*	Tschinkel et al. (1995)
Basal area of mound	Worker number	0.79	30	*F. podzolica*	Savolainen et al. (1996)
Territory area	Colony biomass	0.79	30	*S. invicta*	Tschinkel et al. (1995)
Worker number (MRR)	Worker number	0.77	6	*F. neorufibarbis*	Billick (1999)
Territory area	Worker number	0.76	30	*S. invicta*	Tschinkel et al. (1995)
Removed worker number[a]	Worker number	0.69	76	*M. scabrinodis*	Skórka et al. (2006)
Removed worker number[a]	Worker number	0.66	27	*M. rubra*	Skórka et al. (2006)
Depth of the nest	Worker number	0.61	24	*Cataglyphis cursor*	Clémencet and Doums (2007)
Surface area of the nest dome	Worker number	0.59	59	*F. exsecta*	Liautard et al. (2003)
Surface area of the nest dome	Brood production	0.55	59	*F. exsecta*	Liautard et al. (2003)
Basal area of nest	Alate mass	0.25–0.52	49	*F. podzolica*	Deslippe and Savolainen (1994)

R^2 *R* square, *N* sample size

[a] Number of workers removed by sticks in a given period (see text in "Introduction")

Malham et al. (1991), who counted the number of individuals entering or leaving the colony in a given period. This seemed like a simplified MRR method which omitted the recapturing procedure. The error of the estimated size from this method was high, so it may need to be repeated several times on different days for a more accurate average number (Skórka et al., 2006). Tschinkel et al. (1995) found that territory area was related to the biomass of worker and colony, and the number of workers, but it would be time consuming to use this index for estimation of colony size.

Several species of ants in the *Formica rufa* group (red wood ants) are considered "near threatened" by the International Union for Conservation of Nature and Natural Resources (IUCN, 2011) and are protected by law in many European countries (Bernasconi et al., 2011) because of their strong impact on forest ecosystems (Laakso and Setälä, 1997; Ohashi et al., 2007; Żmihorski, 2010). Complete excavation of nests is therefore not feasible as a routine method for studies which need to estimate wood ant colony size or nest size. We compared the feasibility and the accuracy of several MRR methods and the nest mound volume for estimating the nest size of red wood ants. To seek out the best balance for the five assumptions of MRR method, we applied four methods with different levels of invasiveness and collected recapture data over multiple days.

Materials and methods

Species and location

The choice of model species was *Formica lugubris*, which belongs to the well studied *Formica rufa* group in Europe (Cotti, 1996). *F. lugubris* has both monodomous and polydomous social forms (Bernasconi et al., 2005; Maeder et al., 2005), and is polydomous in Great Britain (Sudd et al., 1977). The experiment was conducted in Inshriach forest in the Cairngorms National Park of Scotland in April 2012. Temperatures ranged from 3 to 11 °C. An area of the forest, approximately 25 ha, planted primarily with Canadian lodgepole pine (*Pinus contorta*) was to be clear felled in summer 2012 in order to restore native woodland flora, so colonies of *F. lugubris* in this area were to be severely disrupted. This made the site appropriate for applying invasive measures to the wood ant nests. A preliminary survey recorded 24 nests in approximately 3 ha along the forest edge of this area and no *F. lugubris* nests in an approximately 8 ha deep-forest area. No other species of wood ants were present. To test our nest size estimation method, we selected 15 nests that provided a wide distribution of nest sizes and were accessible for excavation. The minimum distance between these nests and neighbouring nests was >15 m.

Methods

We applied four MRR methods and a mound volume estimation method to the nests. For our four MRR methods, we marked ants on Day 0, and counted the ratio of marked and unmarked ants on Day 1 and Day 2. For each nest, ants were marked by one person with Pactra® paints (Testors, USA) applied as a dot on the gaster using match sticks in three of the methods and with Brillo® spray leather dye (Moneysworth & Best, Canada) in the fourth. Different colours of paints were used for the four MRR methods for each nest. The colours used for these methods were varied between colonies. Pactra paint has been used to mark many ant species in previous studies (Brown and Gordon, 1997; Fewell, 1990; Fewell et al., 1992; Haight, 2012). Laboratory preliminary tests established that both Pactra paint and spray dye can be retained on the cuticle of *F. lugubris* workers for >2 weeks and do not contribute to ant mortality over this time period.

On-the-Trail method

On Day 0, we used Pactra paint to mark and count foragers passing in either direction along the strongest foraging trail at a distance of 0.3–1 m from the nest, for 15 min. On Day 1 and Day 2, the numbers of outgoing foragers marked and unmarked individuals were counted and recorded by one person along the same trail for 15 min. We counted only outgoing foragers to avoid recounting the same foragers if they left and returned to the nest in a short period.

On-the-Surface method

A different colour of Pactra paint was used to mark workers directly on the nest surface for 15 min, regardless of whether the ants had already been marked by the first colour. The number of marked workers was recorded. Each day for the next 2 days, we did recapturing work on the nest surface using a single visual scan sample, which means that one person scanned the whole nest surface only once to count the numbers of marked (with the relevant colour) and unmarked (not painted with that colour) workers.

After-Disturbing method

To make more workers emerge from the nest, we disturbed the nest by lightly tapping the top of the nest by hand for 5 s. We then marked workers directly on the surface with a third colour of paint for 15 min, regardless of whether they had already been marked by another colour/s. On Day 1 and Day 2, we disturbed the nest in the same way and then counted the number of marked (with relevant colour) and unmarked workers on the nest surface using a single visual scan sample by one person.

Mound-Sampling method

Nests were categorised by approximate mound size (small <20 L; medium, 20–85 L; and large >85 L). On Day 0, an appropriate volume (0.5, 2 or 4 L for small, medium or large nest mound, respectively) of mound thatch containing workers was collected from the south-facing part of nest mound and placed in a small bin. We marked all workers in the bin with spray leather dye, regardless of whether they had already been marked by any Pactra paint, but without obscuring other paint marks, and returned all collected soil and ants to the mound. On Day 1, we collected the same volume of thatch to count the spray-marked and unmarked ants then returned all thatch and ants. The same procedures were conducted on Day 2.

Mound-Volume method

To estimate mound volume, the longest basal diameter, the perpendicular diameter and the height of the nest mounds were measured. If a nest was settled on the slope, uphill height of the nest was used as the height of the nest. Relative mound volume was calculated by multiplying these three dimensions.

Nest excavation

As far as possible, we completed all four MRR methods in the order listed above for each nest; however, we could not complete all four MRR methods for certain nests, especially the On-the-Trail method, because of the absence of ants on trails due to cold weather. We chose 11 of the original 15 nests according to the completeness of our data for each nest and aiming to maintain a wide distribution of nest size. We excavated these 11 nests and counted the actual number of workers. We first removed the thatch of the above-ground parts and counted the workers within the nest material, then dug out the underground chambers of the nest, counting the ants in the soil. We used 12 V car batteries to drive 35 W car vacuums and aspirators for collecting and counting ants individually. After excavating the nests and counting the actual number of ants, we relocated the ants with their nest material out of the area which will be clear felled.

Statistical analysis

For each of our four MRR methods, the estimated nest size was calculated using Bailey's (1951) unbiased modified formula, which is thought to have a better estimate than Lincoln index when marked number is small (Gaskell and George, 1972; Paulson and Akre, 1991; Stradling, 1970): $N = T^*(n + 1)/(t + 1)$, where N is the estimated total number of workers in the nest, T is the number of marked ants, n is the total number in the recapture sample (marked and unmarked workers), and t is the number of marked workers in the recapture sample. We excluded data for which the total number in the recapture sample (n) was smaller than total marked ants (T) in our MRR methods. Due to the temperature limitations on foraging, the on-the-trail data were available on only one day and from only 7 nests. For each of the other MRR methods, we estimated the nest size from the Day 1 and Day 2 data separately, and also took the mean of estimated nest size from the two recapture days for analysis. For each nest, if data from one of days were excluded because the total recapture sample number was too small, data from the other day was used as the mean. We used simple linear regression for the relationship between the estimated nest size and the actual nest size. Bayesian information criterion (BIC) (Schwarz, 1978) and adjusted R^2 were used for comparison and measuring how well a model performs (Bingham and Fry, 2010; Fox, 2008; Seber and Lee, 2003). BIC is considered more appropriate than Akaike information criterion (AIC) (Akaike, 1974) if the sample size is larger than 7 (Fox, 2008; Seber and Lee, 2003), though these two methods gave very similar results when applied to our regression models. All data were transformed by $\log_{10}$ to normalise the distributions, and regressions were conducted with the JMP statistics package (version 6.0.0; SAS institute, Cary, NC, USA).

Results

We found that the After-Disturbing method, in which ants were marked on the nest surface after mild disturbance, was the best MRR method for predicting the actual nest size of *F. lugubris*. Both the estimates of nest size from the mean of 2 days' data and from the Day 2 data significantly predicted the actual nest size, with the mean estimated nest size a particularly good predictor (Table 2; Fig. 1a, c). In addition to the After-Disturbing method, the estimated nest size from the Day 1 data of On-the-Surface method (Table 2; Fig. 1e) and the Day 2 data of Mound-Sampling method (Table 2; Fig. 1i) also significantly predicted the actual nest size. As for other methods, the Mound-Volume method weakly predicted the actual nest size with a borderline significant relationship between relative mound volume and actual nest size ($p = 0.054$, Table 2; Fig. 2). There was no significant relationship between the actual nest size and the estimated nest size from the On-the-Trail method (Table 2).

The actual nest size of these 11 nests ranged from 4,251 to 66,285 ants (mean ± SD = 23,442 ± 25,518). Average percentage of marked workers in recapturing day of four MRR methods ranged from 1.6 to 11.7 % (Table 3). We recorded 3, 7 and 18 queens in 3 of 11 nests. We were not aiming specifically to record queens, and it is likely that more queens were present in these and the other nests, but were not identified due to the quick counting of large numbers of individual ants. Many larvae and eggs were found in the biggest and third biggest nests, respectively. Larger workers, which are repletes with distended gasters, were usually found in the underground part of the nests. Few workers were found in the chambers in the north-facing part of the nest thatch. Nests extended approximately 0.5 m deep underground. Ants marked by spray dye and paints were found deep in the underground parts of nest. Mortality of marked ants was neither observed during the experiment nor seen in the dumping places of nests.

Discussion

We tested four MRR methods involving different levels of disturbance, for estimating nest size and also investigated

Table 2 Results of the linear regression for the relationships between the estimated nest size and the actual nest size from five methods

Method	Days	N	F ratio	p	Relationship	R^2	R^2_{adj}	BIC
On-the-Trail	1	7	0.73	0.43	–	–	–	–
On-the-Surface	Mean	11	1.14	0.31	–	–	–	–
	1	7	6.71	*	$\log_{10} y = 0.5796 \times \log_{10} x + 1.7594$	0.57	0.49	−16.48
	2	8	0.14	0.72	–	–	–	–
After-Disturbing	Mean	11	66.38	***	$\log_{10} y = 1.4874 \times \log_{10} x - 1.5951$	0.88	0.87	−37.03
	1	9	3.39	0.11	–	–	–	–
	2	9	17.17	**	$\log_{10} y = 1.1448 \times \log_{10} x - 0.2789$	0.71	0.67	−23.27
Mound-Sampling	Mean	11	4.57	0.06	–	–	–	–
	1	11	1.93	0.20	–	–	–	–
	2	8	7.91	*	$\log_{10} y = 0.4644 \times \log_{10} x + 2.5583$	0.57	0.50	−18.98
Mound-Volume	–	11	4.91	0.05	$\log_{10} y = 0.5846 \times \log_{10} x + 1.1512$	0.35	0.28	−18.43

y actual nest size, x estimated nest size, N sample size, R^2_{adj} adjusted R square, *BIC* Bayesian information criterion

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$

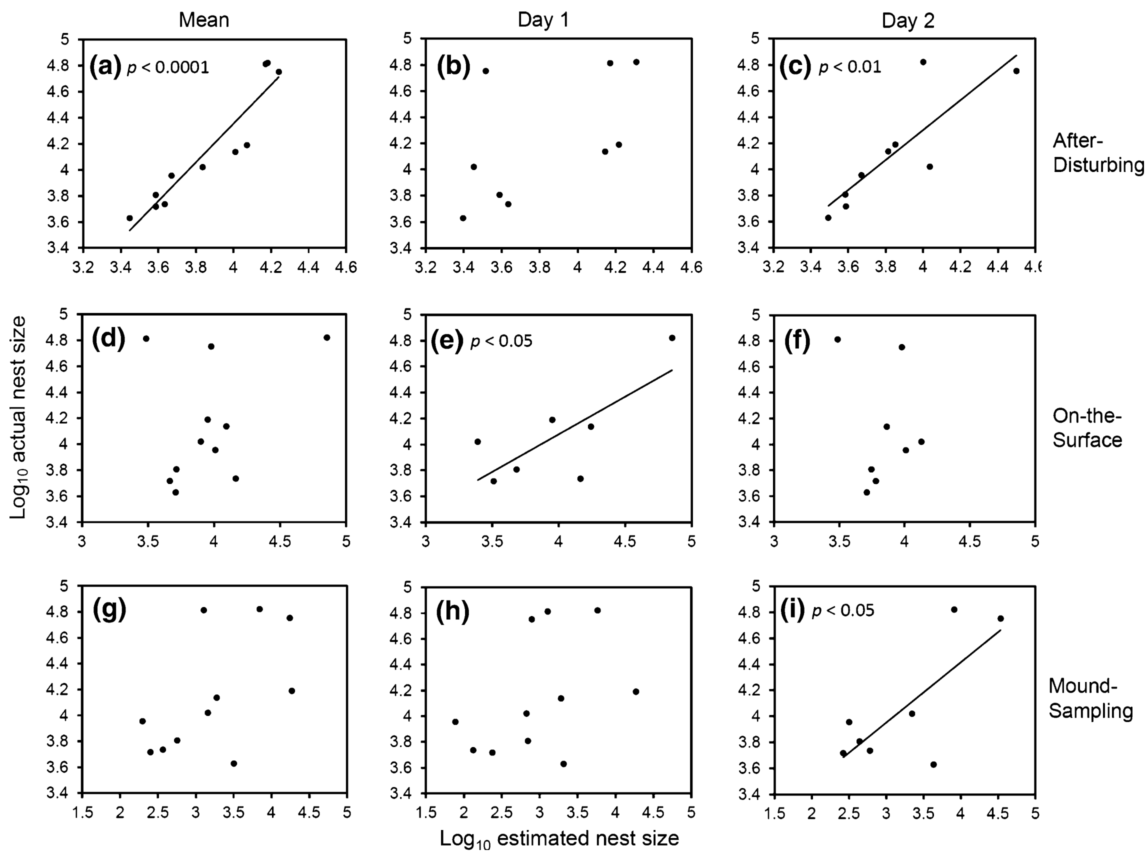

Fig. 1 Relationship between actual nest size and estimated nest size from three mark–release–recapture methods, presenting estimates from Days 1 and 2 of recapture, and also the mean of these estimates (**a–c** After-Disturbing, **d–f** On-the-Surface, **g–i** Mound-Sampling. Regression lines show the significant relationships)

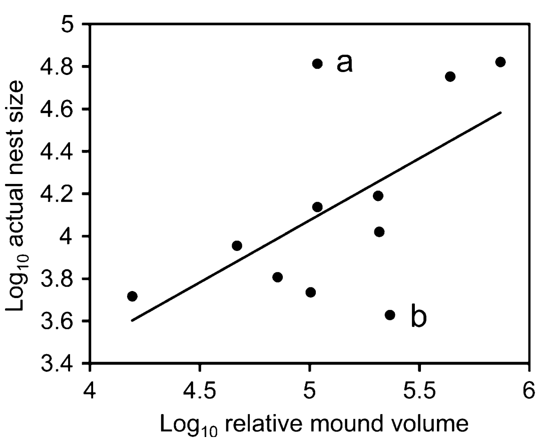

Fig. 2 Relationship between actual nest size and the relative mound volume. *a* and *b* indicate two nests with atypical mound shape (see text for "Discussion")

the relationship between nest mound size and nest size of *Formica lugubris*. We found that our "After-Disturbing" method, in which we performed marking and recapturing after lightly tapping the nest mound surface, was the best method to estimate nest size. Estimates of nest size from this method effectively predict nest sizes ranging from 4,000 to over 60,000 workers. Estimates from the mean of 2 days recapture data contributed to a better fitting regression model than using either day alone, however, estimates from Day 2 data alone may provide the best balance between accuracy and effort, not only because they require less recapture effort but also because the regression model makes a more realistic estimate: in the model of relationship between actual nest size and the estimated nest size from After-Disturbing method in Day 2, parameters of the equation slope and intercept made the regression line the closest to the equation $y = x$, thus with the least underestimating comparing to other methods (Fig. 1).

Lightly tapping the nest mound surface in the After-Disturbing method was probably an important step. Compared to the On-the-Surface method in which workers are not disturbed, adding this step meant that not only can more workers in total be marked and recaptured, but also probably includes workers from other task groups switching to nest defence. Thus, adding this step increases the accuracy of the assumptions, that a representative sample of the colony is taken to mark and estimate, improving the prediction of nest size. In contrast, the On-the-Trail and On-the-Surface methods may only capture certain groups so the estimates

Table 3 Average numbers of marked and recaptured workers for our four MRR methods (mean ± SD)

Methods	Marked number	*N*	Day 1			Day 2		
			Recaptured number	% of marked number	*N*	Recaptured number	% of marked number	*N*
On-the-Trail	93 ± 41	7	116 ± 66	1.6 ± 0.9	7			
On-the-Surface	200 ± 89	11	621 ± 37	3.1 ± 2.7	7	455 ± 217	2.3 ± 1.0	8
After-Disturbing	196 ± 69	11	579 ± 404	3.0 ± 2.2	9	475 ± 275	3.0 ± 1.9	9
Mound-Sampling	110 ± 112	11	327 ± 242	11.7 ± 9.5	11	247 ± 208	6.0 ± 6.5	8

N sample size

from these methods are less likely to represent the whole nest. The same situation may occur in the Mound-Sampling method, in which we only sampled part of the mound. Individuals do not distribute evenly in red wood ant nests (Coenen-Stass et al., 1980), so sampling position within the mound may greatly influence the representativeness of the sampling. Even by lightly disturbing, as we did in After-Disturbing and Mound-Sampling methods, we still cannot capture and recapture all types of workers in a nest. It is likely that this is why all estimates from our four MRR methods underestimated the nest size. However, we found a balance between increased accuracy and reduced destructiveness and time for estimating wood ants nest size, as well as the colony size of monodomous species.

MRR methods require that paint marks are permanent over the time-scale of the study. In laboratory preliminary tests we found that both Pactra Paint and Brillo spray dye can be retained on the workers for more than 2 weeks and do not influence survival. In the Mound-Sampling method, if the ants we captured had been marked by other paint, we sprayed Brillo dye carefully to keep the former mark still visible. Bright and obvious colours such as yellow and pink were used in our experiments and were detected easily. We observed some painted individuals when we were excavating the underground parts of the nests. This sheds light on the assumption of thorough mixing of marked and unmarked workers, suggesting that ants do move through different parts of the nest. Although Porter and Jorgensen (1980) proposed that recapture should be done within 1 day for the minimum effects of high forager mortality in harvester ants, this does not seem to apply to *F. lugubris* and our MRR methods. The significant relationships between actual and estimated nest size of After-Disturbing and Mound-Sampling method from Day 2 data showed that a period of 2 days provided a better estimate, compared to 1 day, suggesting that waiting the extra day allowed colonies to mix more thoroughly. As for the assumption of closed population, our study was conducted during mid-April, at which point there are no pupae and no eclosions in *F. lugubris* (Cherix et al., 2006). The turnover rate of workers was probably low at this period due to the extended longevity caused by winter of the present workers (Calabi and Porter, 1989). The duration of the experiment was also not long enough to cause a considerable change of worker number from births and deaths. Furthermore, although *F. lugubris* is polydomous in Great Britain (Sudd et al., 1977), we chose nests with at least a 15 m distance and without obvious trails between them. Low activity caused by cold weather in April helps to meet the assumption of closed population.

Some previous studies have shown that physical features of the nest such as nest volume, mound volume and basal area of mound can be used to predict the colony size or colony biomass of ants (Table 1) (Mikheyev and Tschinkel, 2004; Savolainen et al., 1996; Tschinkel, 1993; Tschinkel et al., 1995), however, other studies found contrasting results (Breen, 1979; Domisch et al., 2008; Sudd et al., 1977). In our study, Mound-Volume method provided a borderline significant relationship between relative mound volume and actual nest size. Looking into the details of the mound dimensions we found that two outliers in Fig. 2 (points a and b) were from nests with atypical mound shape. Point (a) was from a nest which built the mound on a steep slope. This may have caused us to underestimate the height of the mound. In contrast, a nest which built the mound partly on a fallen tree contributed the data of point (b). This may have caused us to overestimate its height. The relationship between relative mound size and actual nest size would be significant if these two data points were excluded ($F = 19.30$, $p < 0.01$, $N = 9$, $R^2 = 0.73$). In addition, our study was conducted at the beginning of spring, before *F. lugubris* starts to modify the mound (Cherix et al., 2006). Mound volume would be expected to be most stable during this period, when the building work of the previous summer and the decomposition of the previous autumn and winter have had time to stabilise. Therefore, although some studies showed that mound features should not be used as a predictors for the colony size of *F. lugubris* (Domisch et al., 2008; Sudd et al., 1977), we believe that measuring Mound-Volume method can be a feasible wood ant nest size estimation method, at least in the beginning of spring and for nests with relatively typical mound shape. For the nests settled on a slope, the downhill height of the mound is probable a better measurement to calculate relative mound volume rather than the uphill height of the mound.

This study was conducted in April, before colonies became fully active. This posed difficulties for the On-the-Trail method due to cold weather. On the other hand, this season is suitable for the On-the-Surface, After-Disturbing, Mound-Sampling and Mound-Volume methods because the low foraging activity contributes to the isolation of nests and ensures most of the population will be in the nest. It also increased stability of nest population and mound volume compared to other seasons. Because estimates may vary across different seasons due to variation in shading and changing predation rate and food availability, caution should be used if comparing nest sizes across different seasons. However, our method can be applied to the same nest in the same season year by year to monitor the growth or dynamics of nest size. Our After-Disturbing method is probably feasible for many mound-building ant species which would become aggressive and assemble on the mound surface after disturbance of the nest mound. Really counting the exact number of marked and unmarked workers in recapturing may improve the accuracy of our After-Disturbing method. However, the techniques we used, for example, marking individuals on the mound surface and counting by a single scan sample in the recapturing step, can already contribute to an accurate and fast predictor of ant nest size.

To summarise, we found that our After-Disturbing method, in which marking and recapturing were performed after gentle disturbance to the nest, provides a feasible MRR method to estimate colony size for monodomous mound-building ants, or nest size of polydomous species. We improved the method's compliance with the assumptions of MRR by lightly tapping the mound surface before marking and capturing, and conducting the experiment in the beginning of spring. We found that our After-Disturbing method has the best balance between accuracy, non-destructiveness, and time required. We also found that mound volume can be an index of ant nest size under certain conditions. Both methods can be used on the same nest repeatedly to follow the nest dynamics.

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