

## Research



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# Differentiating causality and correlation in allometric scaling: ant colony size drives metabolic hypometry

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Metabolic rates of individual animals and social insect colonies generally scale hypometrically, with mass-specific metabolic rates decreasing with increasing size. Although this allometry has wide ranging effects on social behaviour, ecology and evolution, its causes remain controversial. Because it is difficult to experimentally manipulate body size of organisms, most studies of metabolic scaling depend on correlative data, limiting their ability to determine causation. To overcome this limitation, we experimentally reduced the size of harvester ant colonies (*Pogonomyrmex californicus*) and quantified the consequent increase in mass-specific metabolic rates. Our results clearly demonstrate a causal relationship between colony size and hypometric changes in metabolic rate that could not be explained by changes in physical density. These findings provide evidence against prominent models arguing that the hypometric scaling of metabolic rate is primarily driven by constraints on resource delivery or surface area/volume ratios, because colonies were provided with excess food and colony size does not affect individual oxygen or nutrient transport. We found that larger colonies had lower median walking speeds and relatively more stationary ants and including walking speed as a variable in the mass-scaling allometry greatly reduced the amount of residual variation in the model, reinforcing the role of behaviour in metabolic allometry. Following the experimental size reduction, however, the proportion of stationary ants increased, demonstrating that variation in locomotory activity cannot solely explain hypometric scaling of metabolic rates in these colonies. Based on prior studies of this species, the increase in metabolic rate in size-reduced colonies could be due to increased anabolic processes associated with brood care and colony growth.

## 1. Introduction

A key feature of organismal scaling, and the foundation of the metabolic theory of ecology, is that larger animals use less energy per unit mass [1–7]. While the mass-scaling exponent varies, with few exceptions, it is virtually always hypometric (e.g. two-thirds or three-fourths) and less than the isometric prediction (1.0). This pattern extends beyond individual metazoans to higher biological levels of organization, including social groups and even ecological systems [1–17]. Despite more than a century of empirical research and multiple theoretical models, the mechanistic basis for the hypometric relationship between mass and metabolic rate remains a major unresolved problem in biology [13,18–23].

Many of the models that have been proposed to explain metabolic hypometry rely on the assumption that organismal performance is physiologically constrained at the cellular, organismal or ecological scales by limitations on oxygen or resource intake [7,23–27]. Alternatively, hypometric mass-scaling of metabolism may be determined by variation in demand for ATP [28–30] caused by size-based selection for the performance of specific behaviours affecting metabolism [31–35], or from a mixture of mechanisms that vary across species [23,35]. These alternatives present different and often competing theoretical views of allometric scaling.

One reason for the ongoing controversy lies in the difficulty of generating empirical data that differentiate support for alternative scaling hypotheses. Another major challenge is that the relationships between mass and metabolic rate are almost entirely based on correlative data [9,35–40], making it difficult to evaluate the various causal hypotheses. To empirically test for causality between size and metabolic rate, it would be useful to systematically manipulate mass within an individual entity, and measure the subsequent effects on metabolic rate and the energetic processes affecting metabolism. However, for the vast majority of organisms, this is impossible without sacrificing critical tissues or otherwise inducing physiological trauma.

Colonies of eusocial insects display high levels of cohesion and integration that show parallels with individual organisms. In a highly eusocial colony, reproduction is limited to one or a very few individuals and selection thus occurs primarily at the level of the colony as a whole [10,41–44]. These colonies have strong systems of division of labour and individual task specialization, associated with rich and highly connected communication systems [1,45,46]. Their colony-level development [8,47,48], self-organized behaviours [13,49,50] and patterns of integrated and often homeostatic physiological regulation [51–55] are in many ways functionally similar to individual organisms. As such, they have been termed ‘superorganisms’ [1,14,54,56], and have become useful models for testing questions relevant to organismal organization and function.

Colonies of eusocial insects also generally exhibit hypometric scaling of metabolic rate with colony mass. Sampling both across and within species, eight of 10 studies have demonstrated metabolic scaling exponents significantly less than 1, with an average exponent across all studies to date of 0.76 [57–60]. Hypometric scaling of metabolism disappears if ants are measured as haphazardly collected individuals placed into groups within a chamber rather than as a functioning colony, arguing that hypometric scaling is a property of the integrated superorganism-like colony [1,61–63]. While social insect colonies may exhibit hypometric metabolic scaling for different reasons than organisms, it is possible that common size-based emergent metabolic regulatory principles may extend from cells, to organisms and through complex social groups. Because social insect colonies can be manipulated in size and composition much more easily than organisms, they also provide a system in which to test causal scaling hypotheses across levels of organization.

Prior tests of the effect of manipulation of group size on metabolic rates have found conflicting support for the nature of metabolic rate allometry in animal groups. Among homeothermic social insects, the metabolic rates of cold-exposed honeybee clusters of different sizes scale hypometrically, consistent with their surface area to volume ratios [1,13]; similar patterns have also been shown for cold-exposed huddling mammals (reviewed by [23,64]). However, homeothermic, cold-exposed groups may be a special case because the surface area : volume ratio directly affects the balance of heat loss to heat production; it is difficult to expand such considerations to social groups such as ant colonies that do not endothermically thermoregulate. Among non-social and ectothermic insects, changing group size does not affect the metabolic rate of aggregating caterpillars [28,65]. Experimental tests of size effects have also been conducted by manipulating the size of modular colonial marine organisms [1,31,33,35]. In these systems, reduced size does increase mass-specific

metabolic rates, and this has been explained by relatively increased surface areas associated with feeding and growth [35]. It seems unlikely that similar topological constraints can explain hypometric scaling of metabolic rates in ant colonies as most studies have been performed in the laboratory with ad libitum access to high-quality food.

Many aspects of the complex behaviour of social insect colonies can be directly observed, including the division of labour and allocation of work among individuals within a colony, making them especially useful for dissecting possible mechanisms by which size may affect metabolism [36–38,40]. Many of the hypotheses that have been put forward to explain the hypometric scaling of metabolism in social insect colonies have analogous implications for regulation at the organismal level; these include size-associated changes in: the body sizes of individual workers [10], the distribution of locomotory activity [1], colony density and worker interactions [8], the scaling of surface-to-volume ratios and heat loss from endothermic swarms [13], worker fat content [51] and growth rate or efficiency [1,14]. Importantly, many of these proposed mechanisms can be tested experimentally.

Here, we use experimental manipulation of colony size to test whether changes in metabolic rate observed across colonies of different sizes are caused by size or merely correlate with it. We conduct a controlled size-manipulation experiment on whole colonies of laboratory-reared harvester ants, *Pogonomyrmex californicus* (electronic supplementary material, figure S1). Considering a colony as a single organism with mass ( $M$ ), we model its metabolic rate using a power law with a coefficient ( $a$ ) and an exponent ( $b$ ):

$$\dot{V}O_{2,\text{colony}} = aM^b. \quad (1.1)$$

If genetic, developmental and/or environmental factors are responsible for setting the pace of metabolism, the expectation is that mass-specific colony metabolic rates should not be affected by the manipulation of colony size to half of its previous size. However, if size itself causes the hypometric scaling of metabolism, then colony metabolic rates would scale according to:

$$\dot{V}O_{2,\text{manipulation}} = a\left(\frac{1}{2}M\right)^b. \quad (1.2)$$

Expressed as a ratio, and considering a scaling exponent ( $b$ ), these equations offer a quantitative prediction for the expected increase in mass-specific metabolic rates, independent of the starting mass, following the size reduction:

$$\frac{B_{\text{manipulation}}}{B_{\text{colony}}} = \frac{a((1/2)M)^{b-1}}{aM^{b-1}} = \frac{((1/2)M)^{b-1}}{M^{b-1}} = \left(\frac{1}{2}\right)^{b-1}. \quad (1.3)$$

If there is an ontogeny-independent causal effect of colony size on whole-colony metabolic rate consistent with other scaling studies, we estimate that colonies exhibiting metabolic allometries with exponents in the range of  $2/3$ – $3/4$ , reduced to half their previous size, should exhibit increases in mass-specific metabolic rates of 1.19–1.26 times the rate when measured as intact whole colonies. However, if metabolic rates are specifically associated with inherited variation between colonies or demographic changes in worker size, age or fat content, then experimental reduction of colony size (without also manipulating these variables) should not have systematic effects on average *per capita* metabolic rates.

We also consider possible social and physical mechanisms that might contribute to metabolic scaling. The respirometry enclosures allow tracking of individual and colony-level locomotory behaviours, simultaneously with measurements of metabolic rate (electronic supplementary material, figures S1–S4). Previous studies suggest that work organization and performance change with colony size [57–60] and that work involving foraging, transport and inter-individual contact, requires increased metabolic expenditure [61–63]. Based on prior work demonstrating an effect of colony size on the distribution of walking speeds among individuals in *P. californicus* [1], we predicted that changes in colonial metabolic rates are partially due to changes in locomotory behaviour, with size-reduced colonies predicted to have relatively fewer inactive workers.

## 2. Material and methods

### (a) Experimental design

The primary aim of this study was to determine how size affects the average *per capita* metabolism and locomotory activity within ant colonies. To address these aims, a series of measurements (including metabolic rates, colony masses and activity estimates) were recorded at multiple time points for each colony. The first measurements included a repeated-measures experiment in which colonies were measured twice, once without manipulation (pre-manipulation) and a second time following reduction in size (size reduction). The design of this experiment (electronic supplementary material, figure S1) involved acclimating colonies within a respirometry chamber for 24 h, recording respirometry and video data for 16–24 h, and then censusing the colony to measure the numbers and masses of individuals. Following the mass census, 50% of the worker, larva and pupa populations (by count) were haphazardly removed from the colony and set aside. The reduced-size colony was given 5 days to rest, and then returned to a respirometry chamber to acclimate for 24 h before repeating the respirometry and video recording.

As a control to test for possible stress-induced effects of the size reduction, six colonies were measured an additional two times, following nearly the exact same protocol as for the first experiment. For these colonies, however, after recording the first set of measurements, 50% of the workers, larvae and pupae were removed, set aside and then returned to their colonies after 24 h. As in the size-manipulation experiment, a second set of respirometry measures were conducted 5 days later.

### (b) Rearing ant colonies

All colonies tested were started from queens of the California seed harvester ant, *Pogonomyrmex californicus*. At the start of these experiments, colonies were approximately a year old (351–380 days) and contained two to three queens ( $12.2 \pm 0.4$  mg each). The queens were collected in Pine Valley, CA ( $32^{\circ}49'20''$  N,  $116^{\circ}31'43''$  W, 1136-m elevation) on 2–3 July 2011, just after mating. This population of *P. californicus* exhibits cooperative polygyny, with two to five queens in a single nest; thus the laboratory colonies were initiated from associations of three queens. Colonies were laboratory-reared in a dark incubator set to  $32^{\circ}\text{C}$ . During this time, they were generously fed one to two times per week with grass seeds, frozen fruit flies and droplets of Bhatkar, a synthetic diet for ants [64]. Multiple small test tubes partially filled with water and plugged with cotton were provided at all times to maintain colony humidity. Colony observations and metabolic measures were made in a laboratory in which the ambient temperature ranged from  $28$  to  $34^{\circ}\text{C}$ . None of the colonies produced reproductives during the duration of these experiments.

### (c) Colony size and composition

The number of individuals and their masses for each colony were recorded before and after size manipulation (electronic supplementary material, table S1). Colonies had a mean of:  $33.4 \pm 6.3$  larvae ( $2.9 \pm 0.4$  mg each),  $19.75 \pm 8.5$  pupae ( $4.2 \pm 0.2$  mg each) and  $255.3 \pm 31.0$  workers ( $3.5 \pm 0.1$  mg each). The number of individuals within colonies ranged from 86 to 452 (workers ranging from 70 to 400).

Prior to manipulation, average colony mass was  $1.10 \pm 0.12$  g s.e. and colony mass ranged from 0.32 to 1.70 g. Queen mass was not correlated with colony mass ( $r = 0.21$ ,  $p = 0.53$ ), total larvae mass was nearly correlated with colony mass ( $r = 0.56$ ,  $p = 0.06$ ), total pupae mass was not correlated with colony mass ( $r = 0.21$ ,  $p = 0.52$ ) and total worker mass was strongly correlated with colony mass ( $r = 0.90$ ,  $p < 0.001$ ). The average *per capita* masses of queens, larvae, pupae and workers did not correlate with colony mass ( $r = 0.20 - 0.31$ ,  $p = 0.26 - 0.92$ ).

### (d) Respirometry

Metabolic rates of whole colonies were estimated using flow-through respirometry [65]. To minimize disturbance, colonies were kept in their nest enclosures, which were placed inside and sealed within a respirometry chamber. The chamber lid was transparent to allow video recording of ant colony behaviour simultaneously with respirometry (electronic supplementary material, figures S2 and S3). The respirometry system was designed with push-mode plumbing, with dry  $\text{CO}_2$ -free air supplied by a compressed air tank and regulated at constant flow rate ( $250 \text{ ml min}^{-1}$ ) with mass flow controllers ( $500 \text{ ml min}^{-1}$  max; set to 50%). Air was passed through the reference cell of a  $\text{CO}_2$  analyser and into a multiplexer (RM-8; Sable Systems International, Las Vegas, NV, USA) to automate switching of measurement between baseline and chamber airflows (electronic supplementary material, figure S4). The analyser was calibrated using nitrogen as a zero- $\text{CO}_2$  gas and 11.9 ppm and 298 ppm  $\text{CO}_2/\text{N}_2$  balance gasses to set the span and check linearity. Excurrent air was sequentially passed from the multiplexer through, in order: a Drierite column (indicating Drierite, 10–20 mesh; W. A. Hammond Drierite Co. Ltd, Xenia, OH, USA) to remove water vapour; the sample cell of the  $\text{CO}_2$  analyser; a Drierite/Ascarite column (Ascarite II  $\text{CO}_2$  Absorbent, 8–20 Mesh, Thomas Scientific, Swedesboro, NJ, USA) to scrub  $\text{CO}_2$ ; and the fuel cell of an  $\text{O}_2$  analyser (FC-2; SSI). The flow rate of the excurrent air was periodically checked for accuracy and stability. Lack of back-pressure was confirmed by periodically disconnecting the flow downstream of the  $\text{CO}_2$  analyser and validating that there was no subsequent change in  $\text{CO}_2$  concentration. The temperature of the colony nest enclosure was recorded during each trial using a thermistor fixed to the aluminium base of the respirometry chamber. Data were converted from analogue to digital (UI-2, SSI) and recorded at 1 Hz with Expedata version 1.1.18 (SSI).

Each colony was given fresh food and water, and then placed into a respirometry chamber with ports disconnected from airflow, but open to room air, 24 h prior to the start of respirometry. Although all colonies were measured in chambers of the same size, a prior study demonstrated that ants regulate a constant local density between each other, and that variation in global average density does not explain patterns in colony metabolic rates [1]. Colonies were measured for 16–24 h with repeat-recording enabled so that a file was saved every 1851 s. The multiplexer was digitally controlled by a program in Expedata to automate switching between baseline and respirometry chamber airflow measurements so that each recording included colony respirometry with baseline data both before and after. Following each colony's respirometry run, the wet mass of the colony was determined, counting the total number

of queens, larvae, pupae and workers and weighing the total number of ants in each of these groups to the nearest 0.1 mg.

In six colonies, the CO<sub>2</sub> production by chamber debris was also measured separately to calculate the contribution of debris piles (common in ant colonies) to background production. To do this, the debris and water tubes were removed, placed in a respirometry chamber, and measured using the same method as applied to the colony measurements. The fraction of whole-colony CO<sub>2</sub> production attributable to the debris and water tubes averaged  $0.0027 \pm 0.0004$  (range: 0.0011–0.0044). The mass-specific CO<sub>2</sub> emission rate from the debris in this study was consistent with prior measures of CO<sub>2</sub> emission from debris in which the debris was left in the nest [1], suggesting that invisible microbial biofilms did not contribute significantly to CO<sub>2</sub> emission rate in these nests. Because the measurement error attributable to debris was extremely small and did not scale with colony size ( $F_{1,4} = 1.18$ ,  $p = 0.34$ ), debris measurements were not incorporated into the subsequent measurements or analyses.

### (e) Colony activity

Colony activity patterns before and after size manipulation were analysed from video of colony nest enclosures, measuring the walking speed of all of the workers within each colony. The video acquisition system, previously described [66], enabled repeat recording of high-quality uncompressed AVI video (2024 × 2024 pixels; 15 frames per second). Walking speeds of ants in a colony were estimated by manual tracking of all of the visibly moving ants within segments of video (30 s) which were recorded during the respirometry measurements [67,68]. The tracking data were also used to calculate colony densities, defined as the average inter-individual distance among workers within a nest. This was calculated using pairwise distances between every ant in each of the colonies (electronic supplementary material, figure S9).

### (f) Analysis of metabolic rate data

The data collected included CO<sub>2</sub> and O<sub>2</sub> concentrations of the excurrent airflow and the temperature of the respirometry chamber, recorded as a sequence of baseline-corrected intervals. Although colonies were given 24 h to rest after their nests were placed in the respirometry chamber, they exhibited increased activity following the start of flow-through respirometry; this increase in activity diminished after 2–4 h (electronic supplementary material, figure S5). To determine the time period after which CO<sub>2</sub> emission stabilized, a series of linear regression models were fitted to the time-series respirometry data. This analysis indicated that the respirometry traces had stabilized by 8.7 h. A single metabolic rate was used for each colony recording based on the average of data collected between 8.7 and 14.4 h. Reliable O<sub>2</sub> data (based on signal to noise ratios) were only available for a set of six colonies, so these recordings were used to estimate the colony respiratory quotient ( $0.86 \pm 0.02$  s.e.) and subsequent metabolic rate estimates were based on CO<sub>2</sub> data converted from units of ppm to Watts (oxyjoule equivalent:  $20.39 \text{ J ml}^{-1} \text{ O}_2$ ) and standardized to 25°C assuming a  $Q_{10} = 2.0$  [65].

### (g) Statistical analyses

All statistical analyses were performed in R, version 2.13.1 [69], with additional functions from the *ggplot2* package [70,71]. Where raw scaling data exhibited heteroscedasticity, estimates of the coefficient and exponent in the allometric equation were calculated using both ordinary least-squares and reduced major axis regression on homoscedastic log<sub>10</sub>-transformed data. Reduced major axis regression was conducted using the *lmodel2* package for R [72]. Paired comparisons were evaluated using the non-parametric Wilcoxon signed-rank test. In the results, unless otherwise mentioned, estimates are presented as mean ± s.e. of the means.

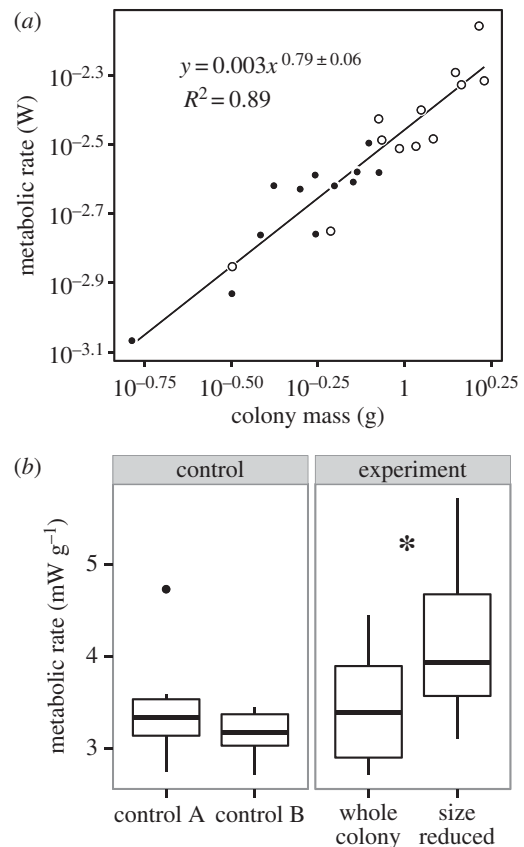


**Figure 1.** The allometric dependence of whole-colony metabolic rate on colony size. (a) On log axes, data for whole-colony wet mass and metabolic rate are plotted with open circles representing colony metabolic rates prior to manipulation and filled circles representing colony metabolic rates after the size reduction. The regression line represents the power-law allometry, which was not significantly different between whole and size-reduced colonies. (b) The experimental size reduction lead to a significant increase in mass-specific metabolic rates (as indicated by an asterisk); there was not a significant effect of the control manipulation on colony metabolic rate.

## 3. Results

### (a) Metabolic rate allometry of unmanipulated and reduced-size colonies

The pre-manipulation and size-reduced measures of whole-colony metabolism exhibited a hypometric scaling relationship with colony size (figure 1a). The allometry was significant ( $F_{1,22} = 175$ ,  $p < 0.001$ ,  $R^2 = 0.89$ ) with a common scaling exponent of  $0.79 \pm 0.06$  s.e. (95% CI: 0.66–0.92) and a scaling coefficient of  $3.48 \times 10^{-3}$ . The reduced major axis model estimated the exponent as 0.84 (95% CI: 0.72–0.97). To determine whether a single allometry was the most appropriate way to describe these data, we used an ANCOVA design to test for an effect of treatment group (pre-manipulation versus size-reduced colonies) on the model parameters and found no significant improvement by fitting different intercepts ( $p = 0.67$ ) or different slopes ( $p = 0.43$ ) for the measurements.

Mean mass-specific metabolic rates for size-reduced colonies were significantly greater than metabolic rates before manipulation (Wilcoxon signed-rank test,  $N = 12$  paired observations,  $V = 11$ ,  $p = 0.023$ , figure 1b). Size reduction led to an increase in mass-specific metabolic rate in nine of 12 colonies, with post-manipulation metabolic rates on average 1.21 times higher than pre-manipulation metabolic rates (95% CI: 1.07–1.35). The magnitude of change in metabolic

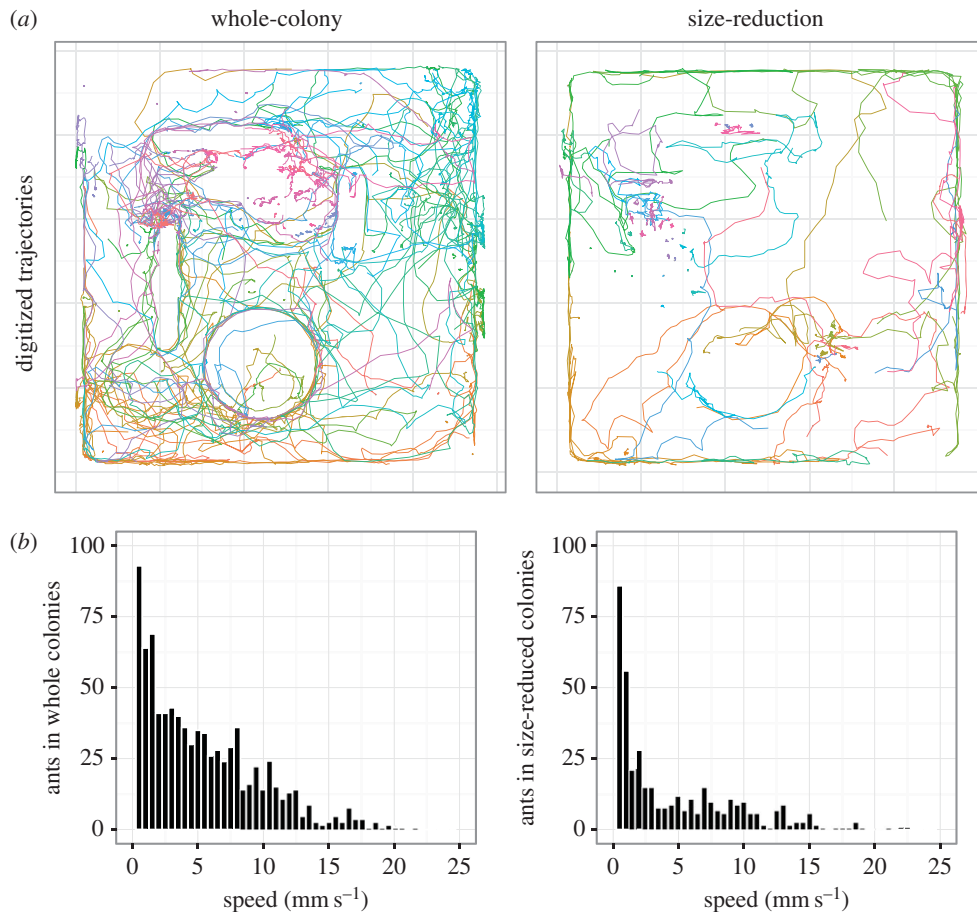


**Figure 2.** Behavioural tracking. (a) The spatial trajectories of all ants were analysed based on video recorded simultaneously with flow-through respirometry. Shown here are the time-aggregated kinematic data from a single colony, originally with 242 workers, and with 123 in the size-reduced state. (b) The speeds measured for all 1443 moving ants (pooled across colonies) followed a highly right-skewed distribution both before and after the experimental size reduction. The histograms do not include the data for ants that were not moving (1129 stationary ants in the whole-colony measurements and 584 stationary ants in the size-reduced colonies). Complete tracking and walking speed data are available in the electronic supplementary material, text. (Online version in colour.)

rates matches the theoretical prediction (1.19) based on equation (1.3), and is significantly greater than the alternative isometric prediction. The average mass-specific metabolic rate of whole colonies was  $3.48 \pm 0.18 \text{ mW g}^{-1}$  and the average mass-specific metabolic rate of the size-reduced colonies was  $4.14 \pm 0.24 \text{ mW g}^{-1}$ . We found no evidence for stress-induced effects of ant-removal and colony handling on colonial metabolic rates (figure 2b; Wilcoxon signed-rank test,  $N = 6$  paired observations,  $V = 17$ ,  $p = 0.218$ ) in our control experiment in which half the individuals (i.e. workers, larvae and pupae) within a set of colonies were removed for 24 h and then returned to their respective source colonies (electronic supplementary material, table S2).

### (b) Changes in movement and activity with colony size

For eight of the 12 colonies, the walking speeds of all ants visible during the two measurements of the size-manipulation experiment were quantified by tracking individual positions during 30-s 15 fps video recordings (3155 ants in total and 283 950 manually digitized coordinates; figure 2 and electronic supplementary material, figure S6 and table S3). Across all colony measures, there was no significant effect of colony size on mean walking speed (linear regression,  $F_{1,14} = 0.44$ ,  $p = 0.52$ ), however, median walking speed did show a negative relationship with increasing colony size among the pre-manipulation colonies measured before experimental size reduction ( $F_{1,6} = 6.74$ ,  $p = 0.04$ ,  $R^2 = 0.45$ ). The average

mean and median walking speeds were  $2.37 \pm 0.37 \text{ mm s}^{-1}$  and  $0.52 \pm 0.26 \text{ mm s}^{-1}$  for the pre-manipulation colonies ( $n = 8$ ), and  $2.07 \pm 0.38 \text{ mm s}^{-1}$  and  $0.39 \pm 0.25 \text{ mm s}^{-1}$  for the size-reduced colonies ( $n = 8$ ). Variation in walking speed explained a significant amount of residual variation in the mass-scaling of metabolic rate across the colonies for which we had both speed and metabolic rate data ( $N = 8$  paired measurements). In this subset of colonies, metabolic rate scaled with  $\text{mass}^{0.72 \pm 0.06 \text{ SE}}$  ( $F_{1,14} = 129.9$ ,  $R^2 = 0.90$ ,  $p < 0.001$ ). Including average walking speed as a second independent variable improved the fit of the allometry model ( $F_{13,14} = 6.45$ ,  $p = 0.02$ , electronic supplementary material, figure S8). With two independent terms, the combined allometry including walking speed ( $v$ ) is an equation of the form:  $\dot{V}O_2 = aM^b v^c$ . The coefficient ( $a$ ) was found to be 0.003, the mass-scaling exponent ( $b$ ) was  $0.74 \pm 0.05 \text{ s.e.}$  and the speed exponent ( $c$ ) was  $0.17 \pm 0.07 \text{ s.e.}$  Together, colony mass and average walking speed accounted for 94% of the variation in colonial metabolic rates.

Owing to relatively high numbers of stationary ants, the distribution of walking speeds within each colony was strongly skewed to the right (electronic supplementary material, figures S3 and S7). Consistent with the hypothesis that the hypometric metabolic scaling exponent reflects the effects of variation in locomotory activity with colony size, the proportion of ants that were inactive (defined as average speed less than  $1.0 \text{ mm s}^{-1}$ ) increased with colony size for both the pre-

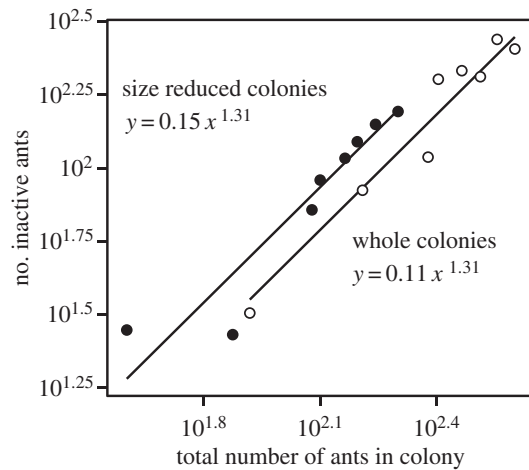


**Figure 3.** Allometric scaling of activity. The relative number of inactive ants increased with colony size within both treatments. The hypermetric exponent (1.31) relating the number of inactive ants to colony size was not significantly different between the whole colonies and size-reduced colonies. Inactive ants were classified as such if their walking speeds were less than  $1.0 \text{ mm s}^{-1}$ .

manipulated and size-reduced colonies, considered separately (figure 3). Expressed as an allometry, the number of stationary ants scaled hypermetrically with colony size, with the number of workers raised to the 1.31 power (95% CI: 1.07–1.55). The size-reduced colonies, however, had relatively more inactive workers (69%) than when they were measured as whole colonies (61%), suggesting that other relatively stationary and metabolically costly behaviours (e.g. egg-laying, food-processing or brood care) were stimulated by the experimental size reduction.

### (c) Changes in density

The *P. californicus* colonies maintained a constant density despite significant variation in the number of workers. The nest containers remained at constant volume; thus, if ants distributed themselves entropically, their density in nests would increase in proportion to their number. However, we found no effect of colony size on the average distance between ants (overall mean =  $108.9 \text{ mm} \pm 3.6 \text{ s.e.}$ ), and tests by both linear regression ( $F_{1,14} = 0.73$ ,  $N = 16$ ,  $p = 0.4$ ) and a repeated-measures *t*-test ( $t = -1.49$ , d.f. = 7,  $p = 0.18$ ) failed to reject the null hypothesis that the average distance between ants was independent of colony size. Thus, consistent with previous findings [1,73], workers regulated their spatial organization to maintain a relatively constant inter-individual spacing and the observed changes in metabolic rate with colony size cannot be attributed to changes in ant density.

## 4. Discussion

This manipulative test of metabolic scaling demonstrates that size can indeed be a causal factor driving hypometric changes in metabolic rate. A reduction in size increased the mass-specific metabolic rate of colonies despite the fact that worker size, brood content, etc., were all kept constant. When size-reduced, colonies exhibited significantly elevated mass-specific metabolic rates, with the magnitude of the increase not significantly different from that predicted by the scaling of metabolism across intact colonies. These scaling effects did not occur within colonies that were similarly manipulated, but not size-reduced, indicating that colony size (rather than

disturbance) was the primary causal variable driving the increase in mass-specific metabolic rates.

Since chamber size was constant across these studies, might the changes in density cause the changes in metabolic rate as found previously for crowded ants [74]? As in our prior study [1], we found that ants maintained constant inter-individual spacing when colony size changed (electronic supplementary material, figure S9). Thus, we can conclude that these experimentally induced metabolic changes were unlikely to be due to changes in physical density. Conceivably, more individuals in the same chamber size might have allowed the accumulation of higher concentrations of volatiles that could influence behaviour and metabolism, but since we maintained air flow rates through the chambers high enough to keep  $\text{CO}_2$  levels below 100 ppm, this seems unlikely.

While previous studies have suggested that demographic variation at the individual-level contributes to scaling effects [10,75], because our colony manipulations controlled for demography, these results strongly suggest that hypometric scaling of metabolic rates in ant colonies (or at least in *P. californicus*) does not require or depend on demographic factors such as changes in the relative numbers of brood or workers of different ages or sizes.

Many of the recently prominent models to explain the hypometric scaling of metabolic rate argue that reduced per-gram energy use by larger organisms reflects increased constraints for resource transport through distribution networks and/or across surface areas [25,26,35]. Such limitations on nutrient delivery seem unlikely here, as these colonies had food available ad libitum that could be collected from foraging areas and returned to the colony within minutes. Furthermore, oxygen levels within nests remained stable and normoxic, so it is unlikely that colony size would have affected oxygen delivery or gas exchange.

Hypometric scaling of metabolic rates have also been hypothesized to be due to size effects on ATP demand [23,76]; one aspect of metabolic demand is locomotor activity. We have previously shown that larger *P. californicus* colonies had more skewed walking speed distributions as well as greater division of labour among workers [1,73], suggesting that colony size-induced changes in worker task performance and locomotory behaviour could explain hypometric scaling of metabolic rate. Differences in walking speeds of workers did significantly contribute to explaining across-colony variation in metabolism, both within unmanipulated and size-manipulated colonies. We found that median speeds decreased in larger colonies, and the proportion of inactive ants increased with colony size within treatment groups. However, compared with pre-manipulated colonies, size-reduced colonies had a relatively greater fraction of stationary ants. Thus, while the decrease in activity in larger colonies is consistent with the hypometric metabolic allometry, variation in locomotory activity level cannot explain the increase in metabolic rate in size-reduced colonies. This finding suggests that future studies should investigate the scaling of other major components of whole-colony energy budgets.

In addition to locomotion, colonial metabolic rate could be affected by anabolic ATP use (protein synthesis and turnover), ATP use associated with ion transport (possibly linked with neuronal activity) or changes in the efficiency of conversion of the chemical energy in metabolic substrates to ATP. There is evidence for *P. californicus* that both size-reduced

and smaller whole colonies have more workers allocated to brood care, suggesting that smaller colonies may have higher growth rates [59,73]. Small colonies are especially vulnerable to predation and raiding, and thus may allocate more time, materials and energy for worker production to generate faster growth and so reach sizes in which they can become more competitive with other colonies and/or species.

We manipulated colonial size and measured it 5 days later in order to provide time for colonies to recover from the stress of the manipulation but not to allow too much time for compensation. Plausibly, the mechanisms responsible for changes in metabolic rate may depend on the time since manipulation; thus, the mechanisms causing changes in metabolic rates might be different in our week-long size manipulation than in naturally growing colonies. For example, colony size may affect worker behaviour and metabolism via effects on larval nutritional experience, and thus understanding the mechanisms of hypometric scaling in unmanipulated colonies may require longer term studies that allow the multiple weeks required for larval development.

What sensory mechanisms might mediate size effects on behaviour and metabolic rate? The ants have some mechanism to determine what their colony size is, and to adjust metabolic rate. While we have excluded physical density, it

is plausible that ants may use visual, olfactory or tactile cues to assess the size of their colony, possibly by some form of counting of total individuals, total interactions or counts of the diversity of stimuli. Alternatively, larger colony size might dilute some chemical signal such as a queen pheromone. Future studies that explicitly manipulate such cues have the potential to reveal fundamental pathways for linking size and metabolism for social insects, and will provide inspiration for investigation of how size affects metabolism in organisms.

**Data accessibility.** The datasets supporting this article have been uploaded as part of the electronic supplementary material.

**Authors' contributions.** J.S.W. conceived of the study, designed the study, ran the experiments, analysed the data and drafted the manuscript; A.O. participated in running the experiment and analysing the data; J.H.F. and J.F.H. participated in the design of the study, provided necessary resources and helped draft the manuscript. All authors gave final approval for publication.

**Competing interests.** We have no competing interests.

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# PROCEEDINGS OF THE ROYAL SOCIETY B

## BIOLOGICAL SCIENCES



**Electronic Supplementary Material:**

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## Online Supplementary Material

Differentiating causality and correlation in allometric scaling: ant colony size drives metabolic hypometry

James S. Waters, Alison Ochs, Jennifer H. Fewell, and Jon F. Harrison

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### Tables:

|           |                                                 |
|-----------|-------------------------------------------------|
| Table S1. | Colony demographics.                            |
| Table S2. | Summary of colony mass and metabolic rate data. |
| Table S3. | Walking speed summary data.                     |

### Figures:

|            |                                                                      |
|------------|----------------------------------------------------------------------|
| Figure S1. | Protocol and timeline of experiments.                                |
| Figure S2. | Respirometry chamber and system design schematic.                    |
| Figure S3. | Image of a <i>P. californicus</i> colony inside its artificial nest. |
| Figure S4. | Whole-colony flow-through respirometry and activity.                 |
| Figure S5. | Respirometry traces and stabilization time.                          |
| Figure S6. | Trajectories of all tracked ants.                                    |
| Figure S7. | Boxplot displaying the distribution of walking speeds.               |
| Figure S8. | Multiple regression allometry model.                                 |
| Figure S9. | Resilience of spatial organization.                                  |

**Table S1.** Colony demographics. The wet mass of each *Pogonomyrmex californicus* colony, before and after the experimental size-reduction, and the breakdown of colony composition by the total number of individuals and wet mass for each caste. Masses are in units of grams.

| State        | Colony  | Mass   | Queens |        | Workers |        | Larvae |        | Pupae |        |
|--------------|---------|--------|--------|--------|---------|--------|--------|--------|-------|--------|
|              |         |        | N      | mass   | N       | mass   | N      | mass   | N     | mass   |
| Whole-colony | pcp1105 | 0.9672 | 3      | 0.0308 | 239     | 0.7986 | 25     | 0.0326 | 24    | 0.1052 |
| Whole-colony | pcp1147 | 1.4032 | 2      | 0.0198 | 400     | 1.3274 | 34     | 0.0441 | 4     | 0.0119 |
| Whole-colony | pcp1107 | 1.1173 | 3      | 0.0308 | 326     | 1.0717 | 14     | 0.0148 | 0     | 0.0000 |
| Whole-colony | pcp1154 | 1.2110 | 3      | 0.0395 | 292     | 1.0178 | 29     | 0.1537 | 0     | 0.0000 |
| Whole-colony | pcp1121 | 1.4602 | 3      | 0.0360 | 360     | 1.2898 | 82     | 0.1344 | 0     | 0.0000 |
| Whole-colony | pcp1123 | 0.3185 | 1      | 0.0123 | 83      | 0.2993 | 2      | 0.0069 | 0     | 0.0000 |
| Whole-colony | pcp1128 | 1.6995 | 3      | 0.0436 | 376     | 1.3794 | 59     | 0.2289 | 14    | 0.0476 |
| Whole-colony | pcp1143 | 1.0793 | 3      | 0.0356 | 254     | 0.9005 | 49     | 0.1432 | 0     | 0.0000 |
| Whole-colony | pcp1114 | 0.8602 | 3      | 0.0349 | 162     | 0.5592 | 38     | 0.0893 | 44    | 0.1768 |
| Whole-colony | pcp1131 | 0.8426 | 1      | 0.0135 | 228     | 0.7516 | 12     | 0.0359 | 9     | 0.0416 |
| Whole-colony | pcp1113 | 1.6416 | 1      | 0.0129 | 273     | 0.9895 | 27     | 0.1078 | 96    | 0.5314 |
| Whole-colony | pcp1101 | 0.6124 | 3      | 0.0406 | 70      | 0.2362 | 30     | 0.1222 | 46    | 0.2134 |
| Size-reduced | pcp1105 | 0.5000 | 3      | 0.0308 | 120     | 0.4010 | 12     | 0.0156 | 12    | 0.0526 |
| Size-reduced | pcp1147 | 0.7115 | 2      | 0.0198 | 200     | 0.6637 | 17     | 0.0221 | 2     | 0.0060 |
| Size-reduced | pcp1107 | 0.5543 | 3      | 0.0308 | 157     | 0.5161 | 7      | 0.0074 | 0     | 0.0000 |
| Size-reduced | pcp1154 | 0.6279 | 3      | 0.0395 | 146     | 0.5089 | 15     | 0.0795 | 0     | 0.0000 |
| Size-reduced | pcp1121 | 0.7302 | 3      | 0.0360 | 175     | 0.6270 | 41     | 0.0672 | 0     | 0.0000 |
| Size-reduced | pcp1123 | 0.1634 | 1      | 0.0123 | 40      | 0.1442 | 2      | 0.0069 | 0     | 0.0000 |
| Size-reduced | pcp1128 | 0.8441 | 3      | 0.0436 | 180     | 0.6604 | 30     | 0.1164 | 7     | 0.0238 |
| Size-reduced | pcp1143 | 0.5524 | 3      | 0.0356 | 126     | 0.4467 | 24     | 0.0701 | 0     | 0.0000 |
| Size-reduced | pcp1114 | 0.4198 | 3      | 0.0349 | 75      | 0.2589 | 16     | 0.0376 | 22    | 0.0884 |
| Size-reduced | pcp1131 | 0.3842 | 1      | 0.0135 | 100     | 0.3296 | 6      | 0.0179 | 5     | 0.0231 |
| Size-reduced | pcp1113 | 0.7891 | 1      | 0.0129 | 130     | 0.4712 | 14     | 0.0559 | 45    | 0.2491 |
| Size-reduced | pcp1101 | 0.3177 | 3      | 0.0406 | 31      | 0.1046 | 15     | 0.0611 | 24    | 0.1114 |

**Table S2.** Summary of *Pogonomyrmex californicus* colony mass and metabolic rate data. The experimental study included measurements of whole colonies and the same colonies after being reduced to 50% of their original size. The control measurements reflect the measurements before (“Control-a”) and after (“Control-b”) a sham manipulation in which 50% of the colony was removed and then replaced prior to the second measurement to test for a stressful effect of the handling and manipulation. Metabolic rates (standardized to 25 deg. C) were converted to units of Watts as described in the main manuscript text.

| Study      | Colony  | State        | Mass (g) | Metabolic rate (W) |
|------------|---------|--------------|----------|--------------------|
| Experiment | pcp1101 | Whole-colony | 0.6124   | 0.001777           |
| Experiment | pcp1105 | Whole-colony | 0.9672   | 0.003075           |
| Experiment | pcp1107 | Whole-colony | 1.1173   | 0.003973           |
| Experiment | pcp1113 | Whole-colony | 1.6416   | 0.006942           |
| Experiment | pcp1114 | Whole-colony | 0.8602   | 0.003254           |
| Experiment | pcp1121 | Whole-colony | 1.4602   | 0.004699           |
| Experiment | pcp1123 | Whole-colony | 0.3185   | 0.001403           |
| Experiment | pcp1128 | Whole-colony | 1.6995   | 0.004824           |
| Experiment | pcp1131 | Whole-colony | 0.8426   | 0.003745           |
| Experiment | pcp1143 | Whole-colony | 1.0793   | 0.003121           |
| Experiment | pcp1147 | Whole-colony | 1.4032   | 0.005097           |
| Experiment | pcp1154 | Whole-colony | 1.2110   | 0.003279           |
| Experiment | pcp1101 | Size-reduced | 0.3177   | 0.001172           |
| Experiment | pcp1105 | Size-reduced | 0.5000   | 0.002349           |
| Experiment | pcp1107 | Size-reduced | 0.5543   | 0.001742           |
| Experiment | pcp1113 | Size-reduced | 0.7891   | 0.003189           |
| Experiment | pcp1114 | Size-reduced | 0.4198   | 0.002402           |
| Experiment | pcp1121 | Size-reduced | 0.7302   | 0.002633           |
| Experiment | pcp1123 | Size-reduced | 0.1634   | 0.000858           |
| Experiment | pcp1128 | Size-reduced | 0.8441   | 0.002622           |
| Experiment | pcp1131 | Size-reduced | 0.3842   | 0.001731           |
| Experiment | pcp1143 | Size-reduced | 0.5524   | 0.002578           |
| Experiment | pcp1147 | Size-reduced | 0.7115   | 0.002461           |
| Experiment | pcp1154 | Size-reduced | 0.6279   | 0.002400           |
| Control    | pcp1101 | Control-a    | 0.9472   | 0.003179           |
| Control    | pcp1105 | Control-a    | 0.7152   | 0.002569           |
| Control    | pcp1114 | Control-a    | 0.5741   | 0.002715           |
| Control    | pcp1128 | Control-a    | 1.6470   | 0.004523           |
| Control    | pcp1143 | Control-a    | 1.0067   | 0.003325           |
| Control    | pcp1154 | Control-a    | 1.1863   | 0.003655           |
| Control    | pcp1101 | Control-b    | 0.9527   | 0.002861           |
| Control    | pcp1105 | Control-b    | 0.7381   | 0.002548           |
| Control    | pcp1114 | Control-b    | 0.6466   | 0.002100           |
| Control    | pcp1128 | Control-b    | 1.5027   | 0.004071           |
| Control    | pcp1143 | Control-b    | 0.9517   | 0.002955           |
| Control    | pcp1154 | Control-b    | 1.0038   | 0.003424           |

**Table S3.** Walking speed summary data for *Pogonomyrmex californicus* colonies. For eight colonies, the spatial coordinates of all individuals were manually tracked both during the whole-colony and sized-reduced experimental measurements to calculate their speeds ( $\text{mm s}^{-1}$ ). Ants that moved with an average speed less than  $1.0 \text{ mm s}^{-1}$  were classified as inactive. The metabolic rate and mass columns reflect data at the whole-colony level (and for convenience duplicate data also reported in Table S2).

| State        | Colony  | Workers | Mean speed | Median speed | Fraction inactive | Metabolic rate (W) | Mass (g) |
|--------------|---------|---------|------------|--------------|-------------------|--------------------|----------|
| Whole-colony | pcp1105 | 239     | 4.076      | 1.750        | 0.456             | 0.003075           | 0.9672   |
| Whole-colony | pcp1107 | 326     | 2.305      | 0.000        | 0.629             | 0.003973           | 1.1173   |
| Whole-colony | pcp1114 | 162     | 3.133      | 0.905        | 0.519             | 0.003254           | 0.8602   |
| Whole-colony | pcp1121 | 360     | 1.225      | 0.000        | 0.764             | 0.004699           | 1.4602   |
| Whole-colony | pcp1123 | 83      | 2.936      | 1.388        | 0.386             | 0.001403           | 0.3185   |
| Whole-colony | pcp1143 | 254     | 0.967      | 0.000        | 0.791             | 0.003121           | 1.0793   |
| Whole-colony | pcp1147 | 400     | 2.555      | 0.000        | 0.638             | 0.005097           | 1.4032   |
| Whole-colony | pcp1154 | 292     | 1.756      | 0.130        | 0.736             | 0.003279           | 1.2110   |
| Size-reduced | pcp1105 | 120     | 3.077      | 0.347        | 0.600             | 0.002349           | 0.5000   |
| Size-reduced | pcp1107 | 157     | 1.092      | 0.000        | 0.783             | 0.001742           | 0.5543   |
| Size-reduced | pcp1114 | 75      | 4.271      | 2.117        | 0.360             | 0.002402           | 0.4198   |
| Size-reduced | pcp1121 | 175     | 1.394      | 0.000        | 0.806             | 0.002633           | 0.7302   |
| Size-reduced | pcp1123 | 40      | 1.695      | 0.457        | 0.700             | 0.000858           | 0.1634   |
| Size-reduced | pcp1143 | 126     | 1.902      | 0.000        | 0.722             | 0.002578           | 0.5524   |
| Size-reduced | pcp1147 | 200     | 1.779      | 0.000        | 0.775             | 0.002461           | 0.7115   |
| Size-reduced | pcp1154 | 146     | 1.312      | 0.229        | 0.740             | 0.002400           | 0.6279   |

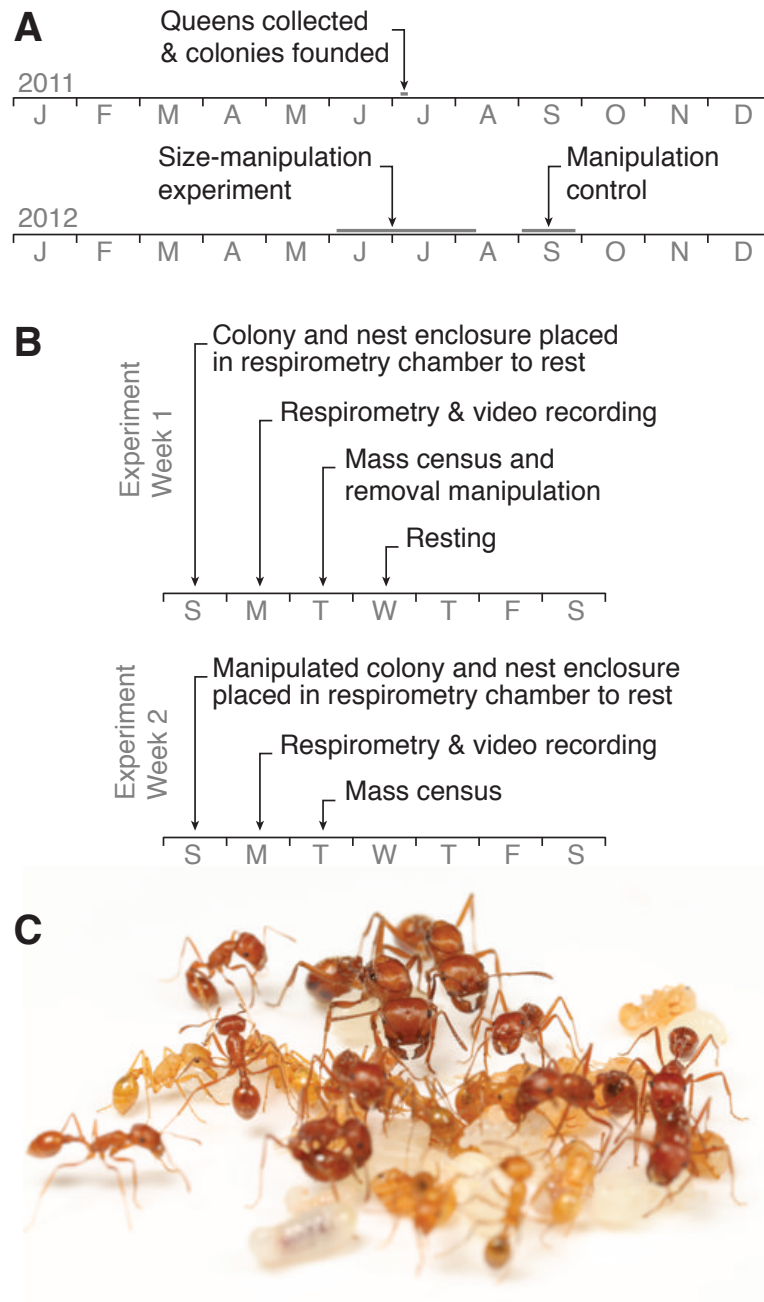


**Figure. S1. Protocol and timeline of experiments.** (A) This timeline displays the sequence of events involved in this study. (B) Details of the schedule for performing the manipulation and control experiments. The control experiment followed nearly the same protocol as the manipulation, except the ants that were removed following the first measurement were returned to the colony one day later. (C) Photograph of a lab-reared *P. californicus* colony composed of queens, pupae, larvae, and workers.

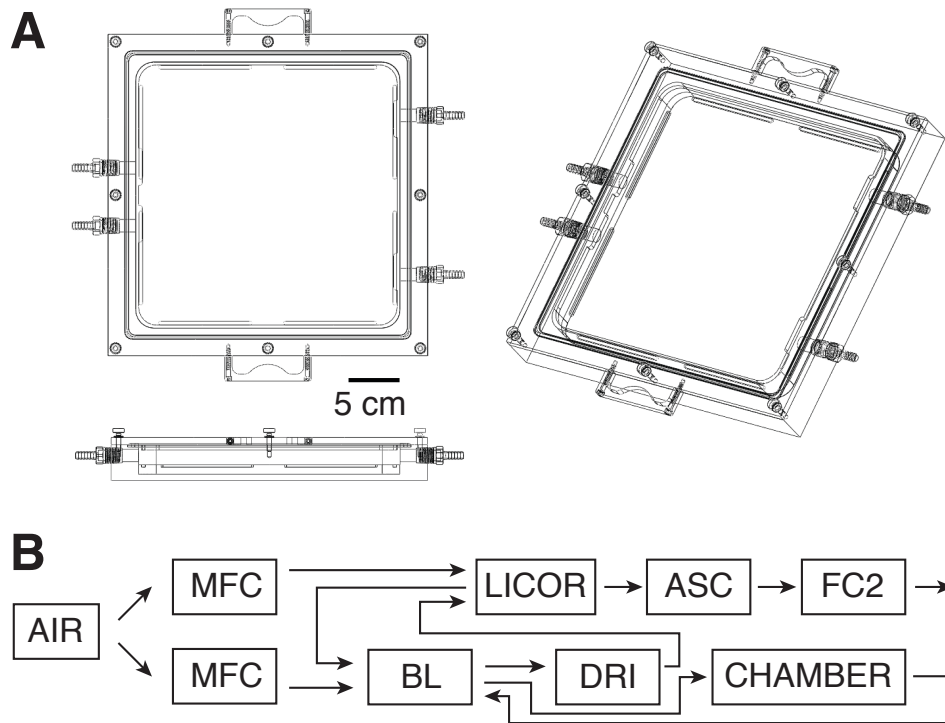


**Figure S2. Respirometry chamber and system design schematic.** (A) The respirometry chamber was constructed with an aluminum base, plexiglass lid, rubber o-ring, and multiple inlet/outlet ports. It holds an artificial nest enclosure made up of a square petri dish (25 cm x 25 cm) with mesh-covered holes on the side placed to align with the respirometry chamber inlet/outlet ports. (B) The airflow was plumbed as a push-model flow-through respirometry system with mass flow controllers (MFC), a baseline multiplexer (BL), CO<sub>2</sub> analyzer (LICOR), Drierite (DRI) and Ascarite (ASC) columns, and an O<sub>2</sub> analyzer (FC2). The baseline multiplexer switches the flow of air into the gas analyzers between the airflow coming from the chamber and a baseline air stream. While the analyzers are baselining, the multiplexer maintains a continuous flow of air into the respirometry chamber from the second mass flow controller avoiding repeatedly disturbing the colony with changes in flow regime.



**Figure S3. Image of a *Pogonomyrmex californicus* colony inside its artificial nest environment and respirometry chamber.** This colony (PCP11-05) had 239 workers. Whole colony sizes in this study ranged from 70-400 workers and the average was 255 workers. This image is a still frame from the video recorded simultaneously with respirometry for the purpose of tracking individual walking speeds. Visible within the nest, in addition to the individual ants, are the water tubes and a petri dish containing food items, a central brood pile, and a refuse pile in the lower right corner. Colonies were reared in the same nest enclosure as they were measured in to minimize disturbance.

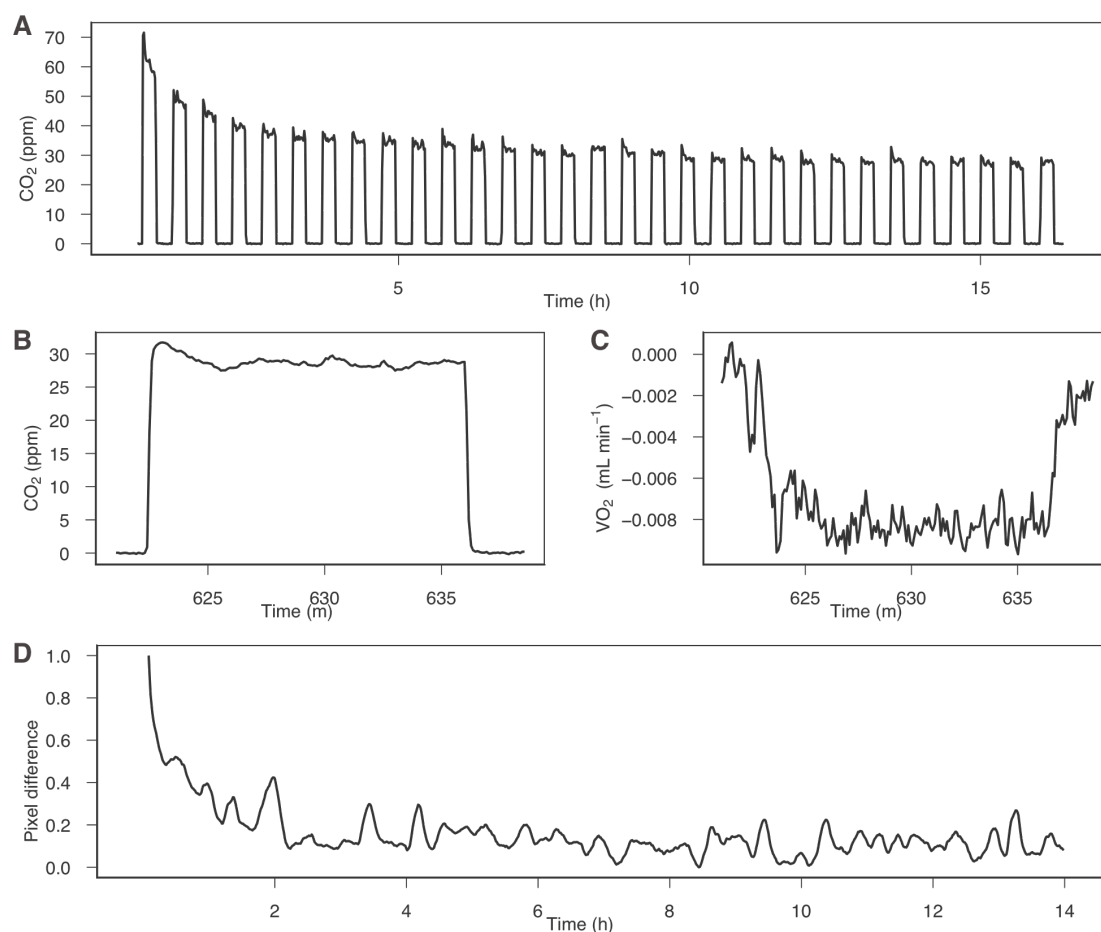


**Figure S4. Whole-colony flow-through respirometry and activity.** (A) A representative sixteen hour respirometry recording, showing alternating switching of airstreams between baseline and the colony chamber during recordings. (B) A single 10 minute  $\text{CO}_2$  recording. (C) A single 10 minute  $\text{O}_2$  recording. (D) Normalized bulk activity metric based on analyzing pixel changes in sequential video frames.

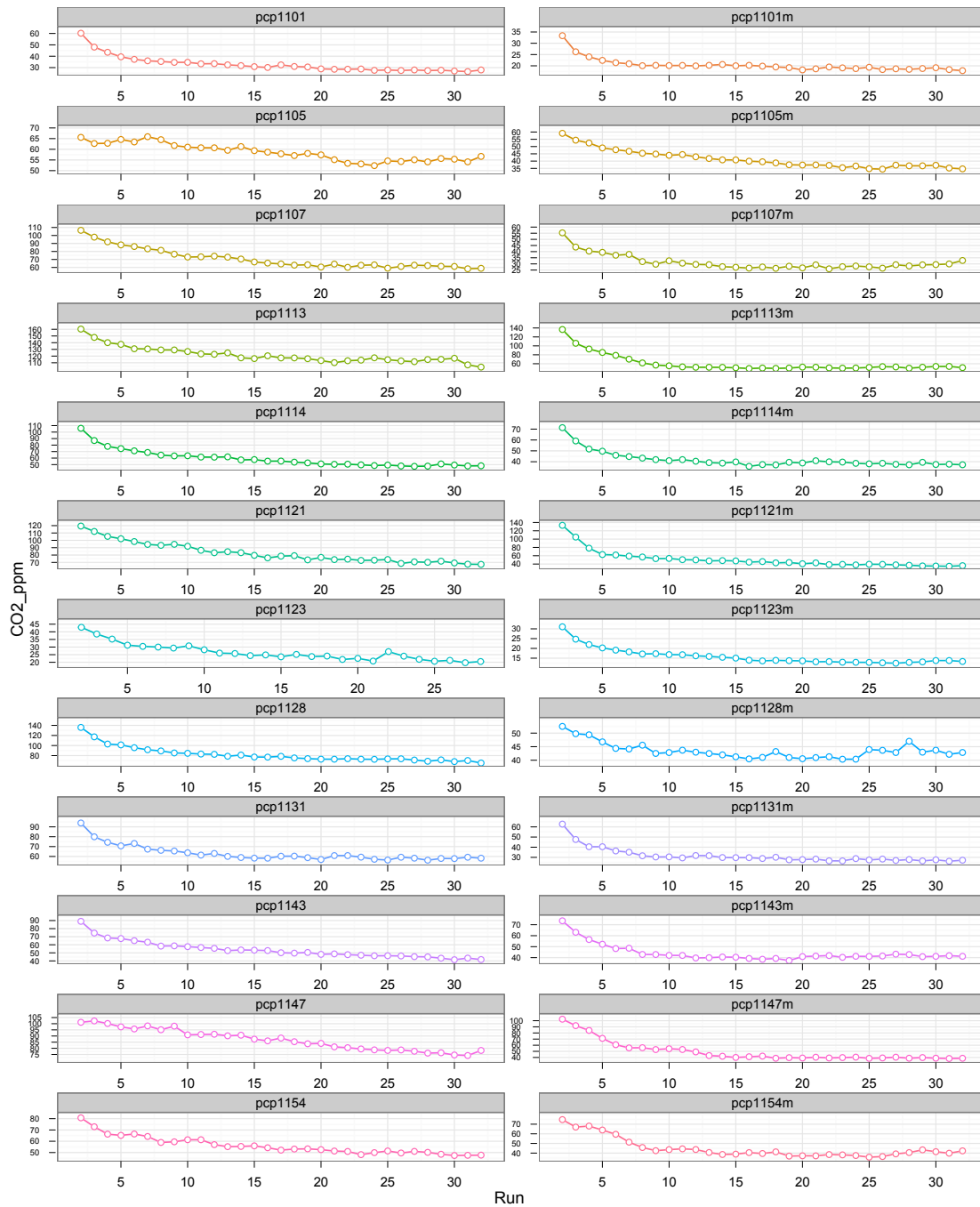


**Figure S5. Respirometry traces and stabilization time.** Shown above are the summaries of hourly respirometry averages across the entire recording duration for all 12 colonies both before and after the size-manipulation.

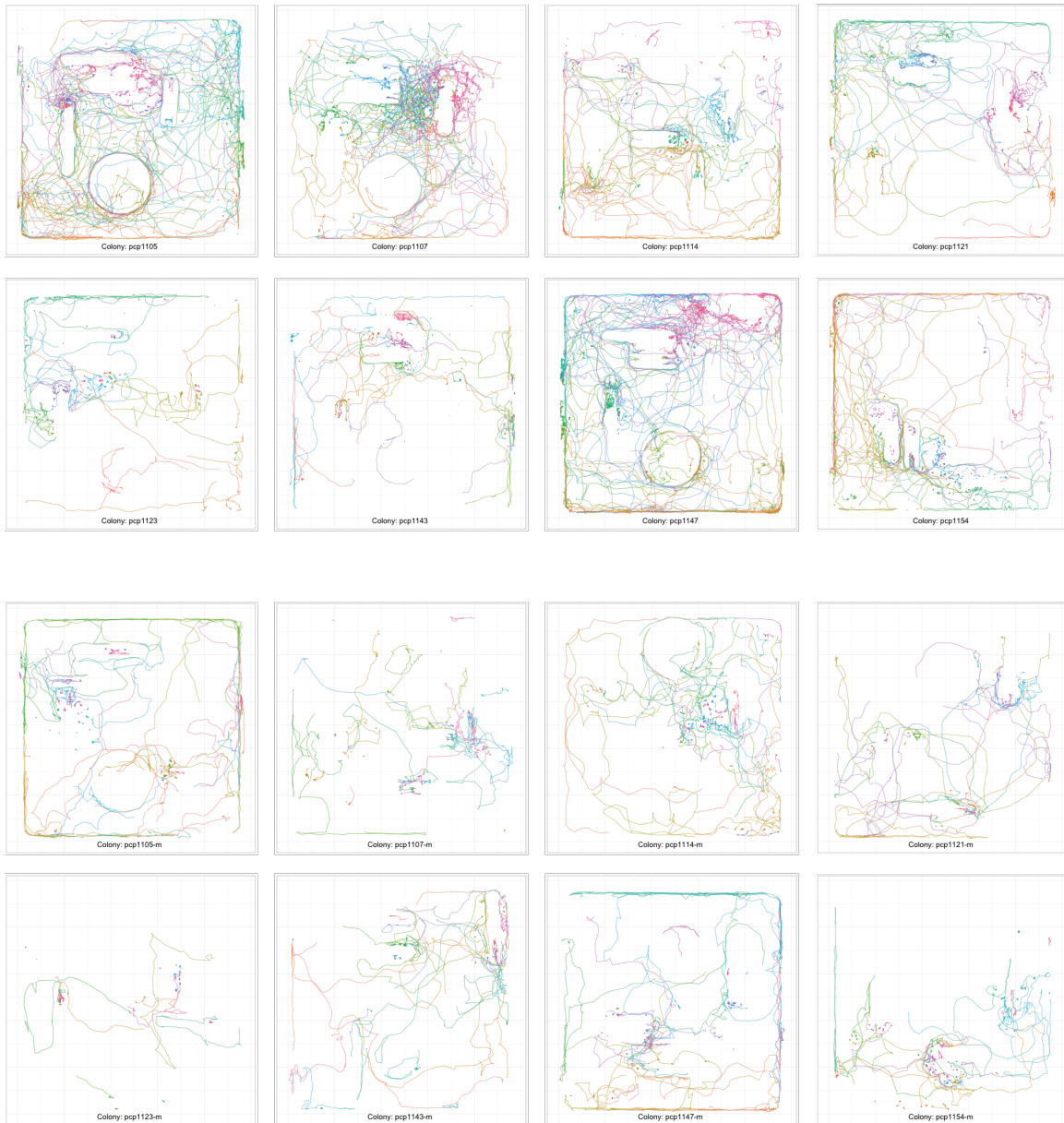


**Figure S6. Trajectories of all tracked ants.** The first eight panels show the trajectories of ants in the whole-colony measurement state; the lower eight panels show the trajectories of ants in the size-reduced colonies.

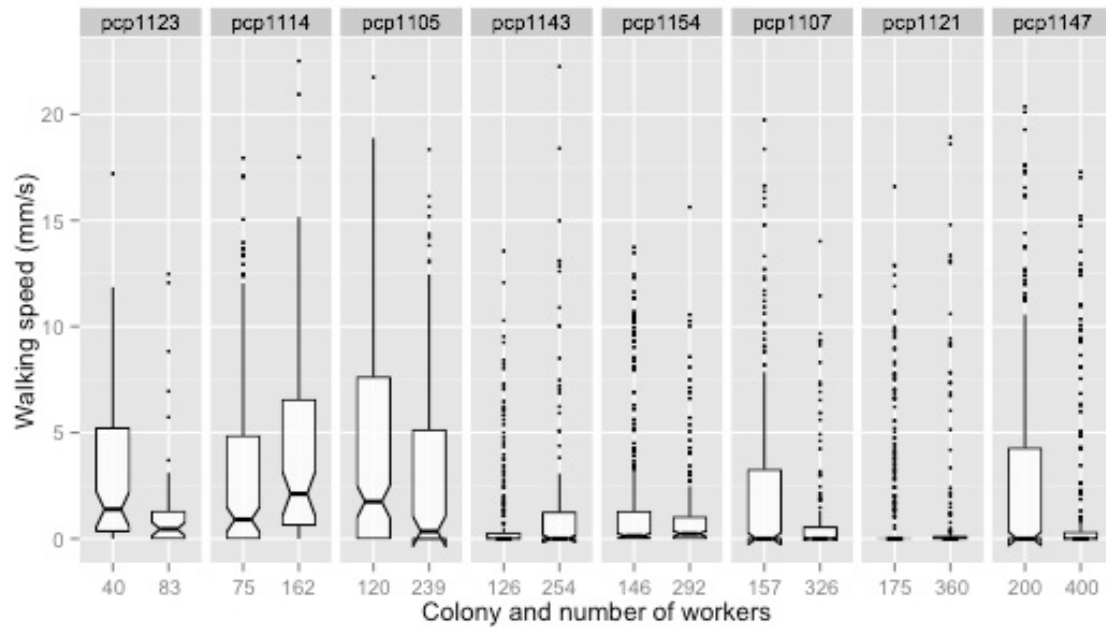


**Figure S7. Boxplot displaying the distribution of walking speeds within and across all colonies and treatments.** The boxes display the median walking speed bounded by the 25th and 75th percentiles, notches indicate the 95% confidence of the median, whiskers extend from the box to the highest and lowest values within 1.5 times the interquartile range, and outliers are plotted for data beyond the whiskers. The data are presented by colony (e.g. “pcp1123” is the name for one colony) and with the number of ant workers present in each case identified on the x-axis.

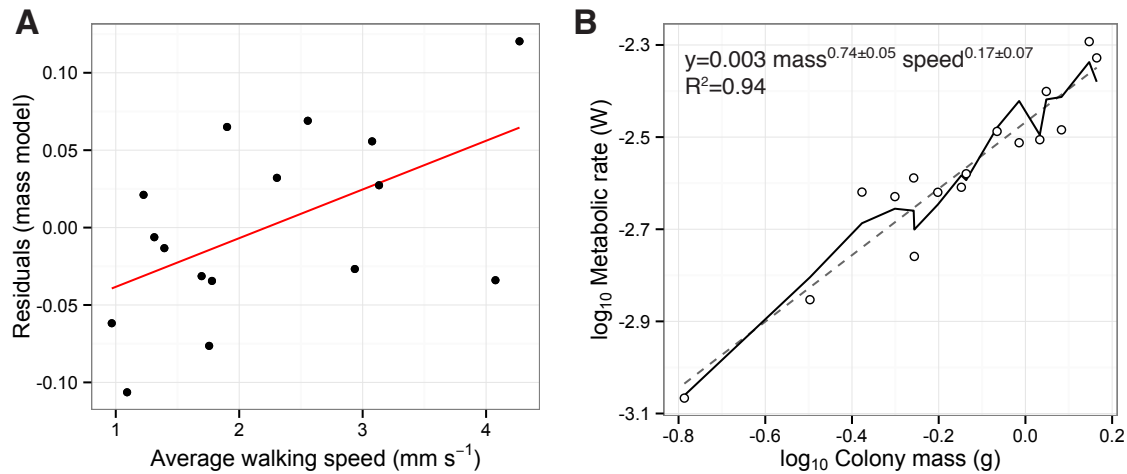


**Figure S8. Multiple regression allometry model.** Whole colony metabolic rates can be modeled as dependent on colony mass and average walking speed. In this case, the model fitting both terms predicts that colony metabolic rate is proportional to the product of mass<sup>0.74±0.05 SE</sup> and speed<sup>0.17±0.07 SE</sup> ( $R^2=0.94$ ,  $p<0.001$ ). This model is a significantly better fit to the data than alternatives including as predictors only either mass ( $p=0.02$ ) or activity ( $p<0.001$ ).

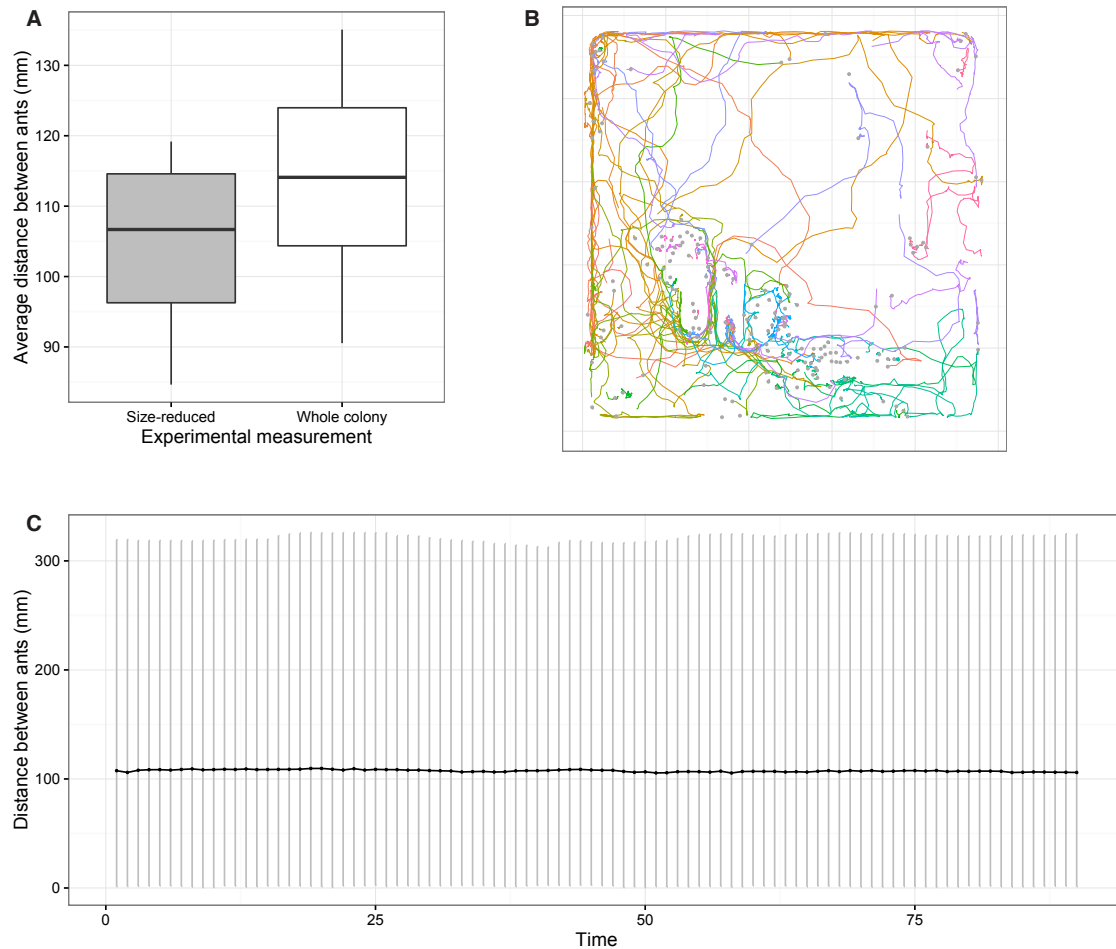


**Figure S9. Resilience of spatial organization.** Despite more than an order of magnitude variation in colony size, the effective density of ants within their (same-sized) nest enclosures was constant. The average distance between ants within a colony (A) was not significantly affected by colony size or the experimental size-reduction. This result was based on calculating all pairwise distances between ants using tracking data (B) collected to quantify variation in speed. The third panel (C) shows a time series of the average distance between ants within a colony with grey vertical bars indicating the full range of distances at each time point. Ants were reared in artificial nest enclosures measuring 250 mm x 250 mm, making the longest distance possible between two ants 354 mm.