



Urbanization influences the trophic position, morphology, and colony structure of invasive African big-headed ants (Hymenoptera: Formicidae) in Taiwan

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Abstract

Ants are among the most prolific invaders especially of urban environments. It has been hypothesized that ants can undergo changes in their behavior, genetics, and phenotype to survive in novel urban habitats. We compared the biology of African big-headed ants (*Pheidole megacephala*) between urban and periurban forest environments in Taiwan. Specifically, we investigated differences in colony structure and morphology between urban and periurban ants in relation to their diet. We further assessed genetic structure of urban and forest *P. megacephala* populations to elucidate the possible role of genetic variation to colony structure and morphology. Urban *P. megacephala* populations exhibited elevated nitrogen stable isotope ($\delta^{15}\text{N}$) values, suggesting a higher trophic position relative to periurban forest habitats; these differences may result from the intake of animal-based resources in urban habitats. Colonies had lower queen number and higher worker relatedness in urban habitats compared with periurban forest habitats. Urban colonies also had larger workers than colonies from periurban populations. Gene flow was not restricted between colonies from urban and periurban environments suggesting that these differences were not strongly influenced by genetic variation among populations.

Key words: Dietary flexibility, invasive ant, genetic adaptation, stable isotope, urban heat island, morphological traits.

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Introduction

Urban areas are often considered hostile environments for arthropod survival because of their impervious asphalt roads and buildings, elevated temperatures due to the urban heat island (UHI) effect, and scarcity of natural food items (FENOGLIO & al. 2020). Nonetheless, urban species have developed alternative strategies for survival in urban environments, including a series of evolutionary changes in the behavior, genetics, and phenotypes of urban populations (DAHIREL & al. 2017, EGGENBERGER & al. 2019). Such changes may exert individual- and colony-level effects, such as altered morphology, colony growth, and reproduction.

Ants are among the most prolific urban invaders (PERFECTO & PHILPOTT 2023). A striking feature contributing to the ecological success of invasive ants is a shift in feeding ecology, which may enable them to use local resources to support massive colonies in disturbed areas. For example, research on the trophic position of ants in south-eastern Australia (GIBB & CUNNINGHAM 2011), South Carolina (RESASCO & al. 2012), and southern Wisconsin (KIM & al. 2019) report that invasive ants generally occupy a lower relative trophic position in disturbed natural areas. This low trophic position is attributed to the high availability of plant-based resources (e.g., nectar and seeds) and a lower availability of prey in disturbed areas. Ants can also eat human food waste, and a study of pavement ants (*Tetramorium* spp.) living in urban habitats in Manhattan reported that the ants exhibited $\delta^{13}\text{C}$ signatures indicating processed human foods (PENICK & al. 2015). In the same study, most ant species (14 species) had median $\delta^{15}\text{N}$ values within or above the isotopic range associated with predators.

The ability of ants to use limited local resources determines their survival and successful reproduction (TILLBERG & al. 2007, WILDER & al. 2011, SUEHIRO & al. 2017). Urban ants exhibit dietary flexibility, whereby they feed on readily available resources rather than on resources that are a part of their natural diet (PENICK & al. 2015). Colony investment in offspring number and size may change in response to colony needs and food availability. In general, ants have diverse species-specific responses to the macronutrient composition. For instance, carbohydrates are crucial for supporting the expansion of colonies, promoting the survival of workers and the brood, and increasing the worker size of globally invasive ants (GROVER & al. 2007, WILLS & al. 2015, WITTMAN & al. 2018), but carbohydrate supplementation significantly reduced the worker size of *Lasius niger* (see GUTIÉRREZ & al. 2020). A review by CSATA & DUSSUTOUR (2019) reported that ants with an imbalanced diet (e.g., a high-protein / low-carbohydrate diet) either (1) consume a small quantity of food to satisfy their protein needs but experience a carbohydrate shortage, or (2) overconsume the imbalanced food to meet their carbohydrate needs but compromise the fitness of the entire colony. Although food supplementation experiments have been widely conducted in invasive ants, many questions remain unanswered, such as how the urbanization pro-

cess modifies the feeding ecology of invasive ants, so that they can obtain the required nutrients for their survival. Recent studies have revealed differences between natural and urban populations of invasive ants in terms of the colony structure (BLUMENFELD & al. 2021) and morphology (FOURNIER & al. 2012). These studies have highlighted the presence of considerable genetic differentiation between populations, suggesting that the urban environment may restrict gene flow and exert intense selection pressure. However, the aforementioned studies have not adjusted for the confounding effects of the diet type on the colony structure and body size.

African big-headed ants (*Pheidole megacephala*) are an invasive species native to Africa, that have been introduced nearly globally (WETTERER 2012). *Pheidole megacephala* is a good model for investigating the responses of ant species to changing environments for numerous reasons. First, *P. megacephala* is common in urban environments and in seminatural and natural forests, making it more resilient to environmental variation than other ant species (MAY & HETERICK 2000, WETTERER 2012, TSAI 2019). Second, *P. megacephala* is a generalist omnivore and is well-known for its dietary flexibility including seeking carbohydrate-rich honeydew (FLUKER & al. 1968). Third, *P. megacephala* exhibits variation in colony structure with large variation in queen number among colonies (BROEKHUYSEN 1948). To date, studies attribute the successful introduction of *P. megacephala* to their interaction with honeydew-producing insects and highly aggressive behavior against other invasive ant species despite their foraging activity being sensitive to changing soil temperature (JAHN & BEARDSLEY 2000, MOTHAPO & WOSSLER 2014, ASFIYA & al. 2016). However, very few empirical studies have focused on food resources, phenotypic evolution, and colony structure of these ants. In-depth studies are essential for identifying the factors (e.g., feeding ecology and adaptive traits) involved in the survival and colony expansion of *P. megacephala* in environments (WETTERER 2012).

We performed stable isotope analyses to compare the trophic niche breadth and trophic position between urban and periurban forest *Pheidole megacephala* populations. Stable isotopes reflect the use of resources over a prolonged period (FELDHAAR & al. 2010). The nitrogen stable isotope ($\delta^{15}\text{N}$) and carbon stable isotope ($\delta^{13}\text{C}$) values were calculated to assess the trophic position and diets of *P. megacephala*, respectively (DENIRO & EPSTEIN 1981, ROUNICK & WINTERBOURN 1986). We had two competing hypotheses. First, that urban *P. megacephala* populations would substantially rely on human food waste rather than on their natural food resources because of the constant availability of human food in urban areas (PENICK & al. 2015). Alternatively, predation would be predominant in the urban environment. Studies have indicated the genetic basis of changes in the colony structure and morphology of ants (FLEATHER 1986, FOURNIER & al. 2009, 2012). We therefore also evaluated the genetic structures of urban

and periurban *P. megacephala* populations to identify any involvement of genetic adaptation in changes in the colony structure and morphology of this species in urban environments.

Material and methods

Study site and sampling

Ants were collected from two study sites – Taiping and Tanzi in Taichung, which is the western region of central Taiwan (24° 04' - 24° 21' N; 120° 35' - 120° 41' E) – between 2018 and 2019. The distance between the two study sites is 6 km. Taichung has a warm, humid, subtropical climate. The air temperature in this area is the lowest in December - March; the monthly maximum and minimum temperatures are 20.1 °C and 17.0 °C, respectively. By contrast, the highest air temperature is recorded in June - October; the monthly maximum and minimum temperatures are 28.9 °C and 25.5 °C, respectively. Precipitation predominantly occurs in summer; the annual average precipitation is 1773 mm (data from Taiwan Central Weather Bureau: <https://www.cwb.gov.tw/V8/C/>).

At each study site, sampling was performed in the hot (August - October) and cold (February - March) seasons for the two habitat types: urban areas (3.01 and 5.32 km² in Taiping and Tanzi, respectively) and periurban forests (1.29 and 2.32 km² in Taiping and Tanzi, respectively). Urban areas comprised pedestrian and urban parks with playground facilities. In these areas, *Pheidole megacephala* colonies are typically found in parks or under isolated street trees. Periurban forests are disturbed forests with agricultural activities. The sites mainly feature trees (logs) such as chinaberry (*Melia azedarach*), cockspur coral (*Erythrina crista-galli*), and Indian almond (*Terminalia catappa*) as well as herbaceous plants such as orange jessamine (*Murraya paniculata*), giant elephant's ear (*Alocasia odora*), and beggarticks (*Bidens alba*). The sites also have some concrete and asphalt hiking trails. *Pheidole megacephala* colonies were found in rotten wood surrounded by trees, which provide canopy cover and leaf litter. In urban areas, ant colonies were sampled under tree roots along the streets or at the park, whereas in the periurban forests, ant colonies were sampled from dead tree logs along forest paths. The distance between two sampled colonies was at least 100 m. To ensure that the colonies sampled originated from different parent colonies, worker ants collected from each colony were transported to the laboratory for aggression tests, which were performed as described by HOLWAY & al. (1998). Only minor workers were selected for analysis because of their abundance in each colony.

To determine the differences in *Pheidole megacephala* diet between urban areas and periurban forests, 13 and 16 colonies from urban areas and 21 and 17 colonies from periurban forests in Taiping and Tanzi were sampled. A sweep net was also used to collect other arthropods from the same sites, which may be potential food sources of *P. megacephala*. To set the autotrophic baseline of the local food web, leaves from the common plants at each study site

such as butterfly needle (Asteraceae: *Bidens pilosa*) and Japanese banana (Musaceae: *Musa formosana*) were used for comparison. This approach was adopted to account for the variations in in situ resource availability and baselines; otherwise, inaccurate ecological inferences would be obtained for the studied systems (HETTE-TRONQUART 2019, KJELDGAARD & al. 2021, MATICH & al. 2021). To increase the statistical power and improve the accuracy of our interpretation, at least five arthropod samples were included in each trophic group (plant producer, herbivores, and predators) (KJELDGAARD & al. 2021). The samples were transported to the laboratory for further species sorting. *Pheidole megacephala* samples were identified using SARNAT & al. (2015). The arthropod samples were identified at the family level by using the identification methods (for insect samples) described by JOHNSON & TRIPLEHORN (2004) and an unpublished identification key developed by spider taxonomists (I.M. Tso, unpubl.) at the Department of Life Science, Tunghai University. The samples were maintained in glass vials at -20 °C until further analysis.

Stable isotope analysis

Stable isotope analysis was performed to measure the ratio of carbon and nitrogen in the tissues of the collected ant samples. All samples were dried at 50 °C for three days. For each colony, the whole body of *Pheidole megacephala* ants (five to six individuals) and other arthropods (weight ~0.5 mg) were packed into tin capsules before the analysis of carbon and nitrogen isotopes. Large arthropod samples and plant specimens were first ground into powder after drying. Then, 0.5 mg of the powdered arthropod samples was packed into tin capsules for carbon and nitrogen isotope analysis. Moreover, 0.5 and 5 mg of powered plant samples were packed into tin capsules for carbon and nitrogen isotope analysis, respectively. The analysis of stable ¹³C and ¹⁵N isotope abundance was performed at the Research Institute for Humanity and Nature, Kyoto, Japan, on the DELTA V Advantage mass spectrometer, which was coupled to a Flash elemental analyzer (EA 1112 series) through the ConFlo IV system (Thermo Fisher Scientific, Waltham, MA). The relative abundance of the stable isotopes was calculated using the following formula:

$$\delta X = (R_{\text{sample}} / R_{\text{standard}}) - 1$$

where δX is $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$, R_{sample} is the $^{13}\text{C} / ^{12}\text{C}$ or $^{15}\text{N} / ^{14}\text{N}$ ratio of the samples, and R_{standard} is the $^{13}\text{C} / ^{12}\text{C}$ ratio of Vienna Pee Dee Belemnite or the $^{15}\text{N} / ^{14}\text{N}$ ratio of atmospheric nitrogen (BARRIE & PROSSER 1996). The $\delta^{13}\text{C}$ value was calibrated using the laboratory standards CERKU-02, CERKU-03, CERKU-05, and CERKU-07, whereas the $\delta^{15}\text{N}$ value was calibrated using the laboratory standards CERKU-02, CERKU-04, and CERKU-05 (TAYASU & al. 2011). The standard deviations in the repeated measurements of the laboratory standards were approximately 0.08‰ for $\delta^{13}\text{C}$ and 0.64‰ for $\delta^{15}\text{N}$.

Colony structure determination and microsatellite genotyping

The colony structure of *Pheidole megacephala* populations was analyzed to evaluate the degree of polygyny in the ant colonies sampled from the urban and periurban forest habitats. For this, destructive sampling and microsatellite genotyping were performed. From the colonies sampled for stable isotope analysis, 10 colonies from urban areas and six colonies from periurban forests were selected and carefully opened to collect female reproductive individuals and workers. To ensure the comprehensive sampling of female reproductive individuals, sampling was performed within a circular area with a radius of 2 m from the main nests. The ants were collected and transported to the laboratory for further sorting and microsatellite genotyping. Eight individuals from each colony (six individuals from colony TPF202) were selected for microsatellite analysis. Genomic DNA was extracted using the Genra Puregene Tissue Kit (Qiagen, Valencia, CA, USA). DNA samples were stored at 4 °C until further analysis.

Seven dinucleotide-repeat microsatellite loci (Pmeg-06, Pmeg-07, Pmeg-09, Pmeg-10, Pmeg-11, Pmeg-12, and Pmeg-14) were selected for microsatellite analysis (FOURNIER & al. 2008). Polymerase chain reaction (PCR) was performed using a reaction mixture (25 μ L) comprising the template DNA (2 μ L), EmeraldAmp Max PCR

Master Mix (12.5 μ L; Takara Bio, Kusatsu, Shiga, Japan), relevant forward and reverse primers (0.2 μ M), and ddH₂O. Multiplex PCR was performed in two groups. The first group comprised Pmeg-06, Pmeg-09, Pmeg-12, and Pmeg-14, with the following PCR cycle: initial denaturation at 95 °C for 15 min; 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 90 s, and extension at 72 °C for 60 s; and final extension at 60 °C for 30 min. The second group comprised Pmeg-07, Pmeg-10, and Pmeg-11, with the following PCR cycle: initial denaturation at 95 °C for 15 min; followed by 35 cycles of denaturation at 30 s, annealing at 56 °C for 90 s, and extension at 72 °C for 60 s; and final extension at 60 °C for 30 min. All PCR products underwent fragment analysis at Genomics BioSci and Tech (Taipei, Taiwan), which was performed using the ABI 3730XL DNA Analyzer (Applied Biosystems Inc., Waltham, MA, USA). GeneMarker (version 2.6.0; Soft-Genetics LLC, State College, PA, USA) was used for allele visualisation and scoring.

Morphological trait measurement

Morphological traits were measured to evaluate the body size of ants in both urban areas and periurban forests. In Taiping, for morphological analysis, 30 minor workers of *Pheidole megacephala* were collected from 4 colonies and 3 colonies in urban area (n = 120) and periurban forest

Tab. 1: Results of generalized linear mixed-effects analysis for the individual effects of habitat, season, and site on the stable isotope signature of *Pheidole megacephala* sampled in Taiping and Tanzi.

Variables	$\delta^{13}\text{C}$			$\delta^{15}\text{N}$		
	Statistical values	P	df	Statistical values	P	df
Habitat	4.457	<0.001	120	10.920	<0.001	120
Season	0.229	0.820	120	-1.379	0.173	120
Site	0.400	0.690	120	1.881	0.064	120

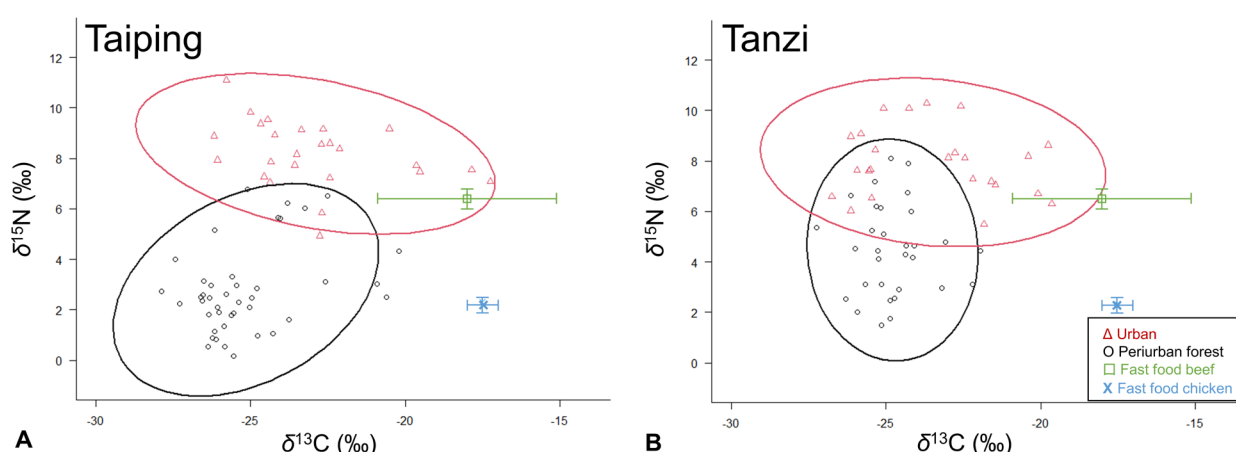


Fig. 1: Biplot of the carbon and nitrogen isotope ratio in Taiping and Tanzi showing the $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values of *Pheidole megacephala* with Bayesian ellipse (curved lines) containing 95% of the data of the urban (red open triangles) and periurban forest (black open circles) populations. The values of fast-food beef (green open square) and chicken (blue cross) were adopted from a study conducted in the United States (JAHREN & KRAFT 2008) because Taiwan imports 70% and 60% of the total beef and chicken for consumption purposes.

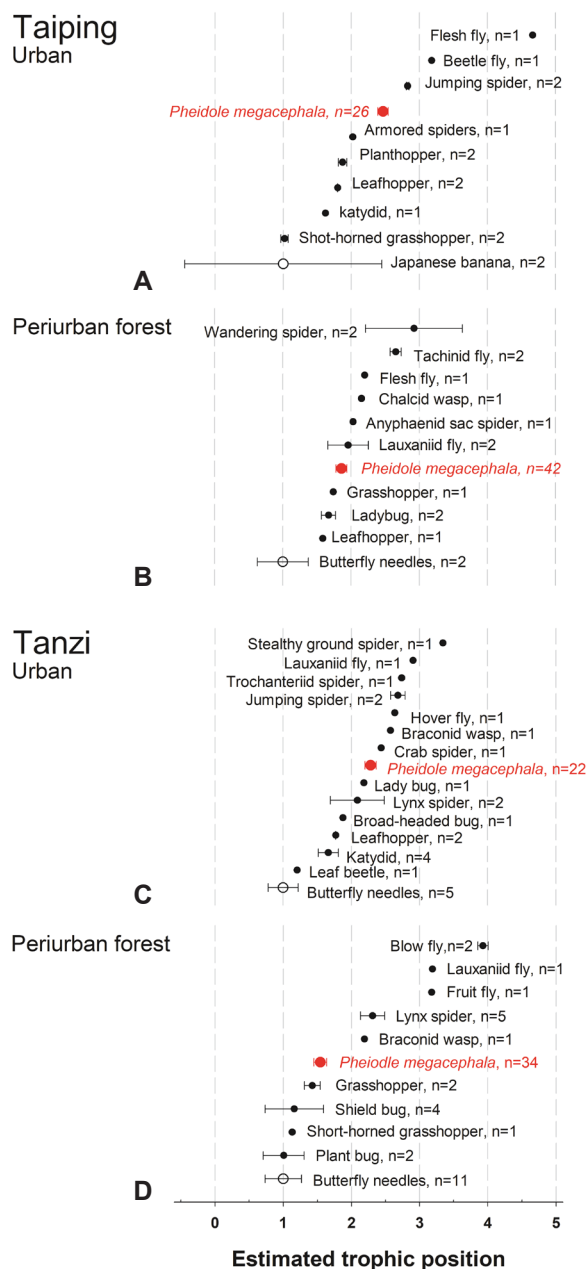


Fig. 2: Estimated trophic levels for *Pheidole megacephala* collected from urban areas and periurban forests in Taiping and Tanzi. Each dashed line indicates a trophic level. The first dashed line delimits the autotrophic baseline of the food web (white). The trophic position of *P. megacephala* is indicated in red. Error bars represent the standard error of the mean values.

($n = 90$), respectively. The following four morphological traits with specific functional roles were measured: head width, which indicates size of gaps through which worker can pass and is also considered as a surrogate for worker size (SARTY & al. 2006); Weber's length, which indicates the body size, metabolic function, and resource use of an individual (KASPARI & WEISER 1999); intereye distance, which may influence the performance and adaptation of visual predators in complex habitats (FOWLER & al. 1991); and mid-femur length, which is associated with the efficiency

and speed of foraging in leaf-litter habitats and the size of crevices that individuals can pass through (KASPARI & WEISER 1999). Morphological traits were measured using S8 APO (Leica Microsystems GmbH, Wetzlar, Germany) and analysed using Helicon Focus (version 6.7.1 Lite; Helicon Soft Ltd., Kharkiv, Ukraine).

Statistical analysis

Statistical analysis was performed in two steps. First, generalized linear mixed-effects models (GLMMs) were used to evaluate the effects of urbanization, season, and sample site on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. Colony IDs were regarded as random effects, whereas habitat and season were regarded as fixed effects. A Gaussian distribution was adopted to model the errors in the analyses of the response variables ($\delta^{13}\text{C}$ and $\delta^{15}\text{N}$). The GLMM analysis was performed using the lme4 package (version 1.1 - 23) (BATES & al. 2015). An analysis of isotope data with Bayesian statistics was performed using the SIBER package (version 2.1.5) (JACKSON & al. 2011). The trophic niche breadths of *Pheidole megacephala* were inferred from the isotopic space occupied by each sample area on a $\delta^{13}\text{C}$ - $\delta^{15}\text{N}$ biplot, which was calculated as Bayesian ellipse areas containing 95% of the corresponding data. The trophic position was estimated as follows:

$$\text{Trophic position} = 1 + (\delta^{15}\text{N}_{\text{ant}} - \delta^{15}\text{N}_{\text{base}}) / 3.4\text{‰}$$

where $\delta^{15}\text{N}_{\text{base}}$ is the mean value of the $\delta^{15}\text{N}$ of plant samples collected from each study site, and 3.4‰ is the mean trophic fractionation of $\delta^{15}\text{N}$ in terrestrial ecosystems (POST 2002). The difference between urban areas and periurban forests in terms of the trophic position of *Pheidole megacephala* was assessed using an independent Student's t test.

Second, the differences in the morphological traits of *Pheidole megacephala* between urban areas and periurban forests were determined using a GLMM; habitats were regarded as fixed effects, whereas colony IDs were regarded as random effects. A Gamma distribution was used to model the response variables (morphological traits) because the data are continuous but bounded as non-negative values. All analyses were performed using R (version 4.0.2) (R CORE TEAM 2020). A P value of < 0.05 indicated statistical significance.

Genetic analysis

The allelic diversity of microsatellite loci was compared between the urban and periurban forest populations of *Pheidole megacephala* in terms of the number of alleles (N_A), allelic richness (A_r), expected heterozygosity (H_E), and observed heterozygosity (H_O). GenAlEx (version 6.5) (PEAKALL & SMOUSE 2012) was used to evaluate N_A , H_E , and H_O for every locus and habitat. A_r was calculated using FSTAT (GOUDET 2001). Urban areas and periurban forests were compared in terms of A_r , H_E , and H_O by using independent Student's t test ($\alpha = 0.05$) and in terms of N_A by using the Mann-Whitney U test.

Tab. 2: Genetic diversity within seven microsatellite loci of *Pheidole megacephala* sampled in Taichung (n, total number of individuals; N_A , number of alleles; Ar, allele richness; H_E , expected heterozygosity; H_O , observed heterozygosity; F_{ST} , fixation index; F_{IS} , inbreeding coefficient).

Habitats	n	N_A	Ar	H_E	H_O	F_{ST}	F_{IS}
Urban	80	4.000	3.663	0.538	0.344	0.296	0.097
Periurban forest	46	3.714	3.698	0.458	0.400	0.023	0.0116

Tab. 3: Results of the analysis of molecular variance test performed using the microsatellite data of *Pheidole megacephala* sampled in Taichung.

Source	df	MS	Percentage of variation	F value	P
Between urban areas and periurban forests	1	23.998	6%	0.057	0.001
Among colonies within habitats	14	9.803	25%	0.262	0.001
Within colonies	236	1.487	69%	0.304	0.001
Total	251		100%		

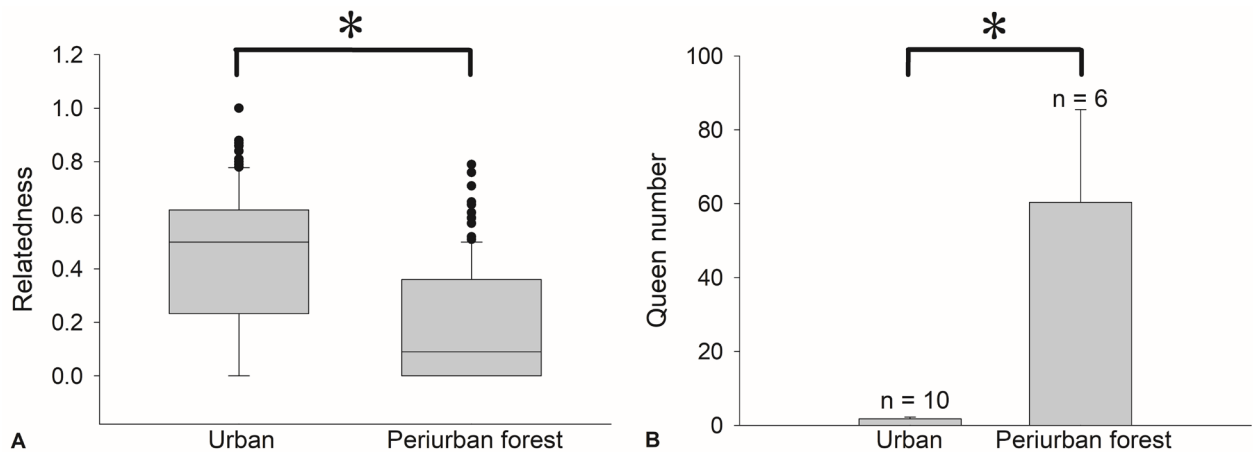


Fig. 3: Colony structure of *Pheidole megacephala* across urban areas and periurban forests. Within-colony genetic relatedness was significantly higher in urban areas than in periurban forests. The mean number of queen ants in *P. megacephala* colonies was significantly lower in urban areas than in periurban forests. Error bars represent standard errors. Asterisks indicate significant difference ($P < 0.05$).

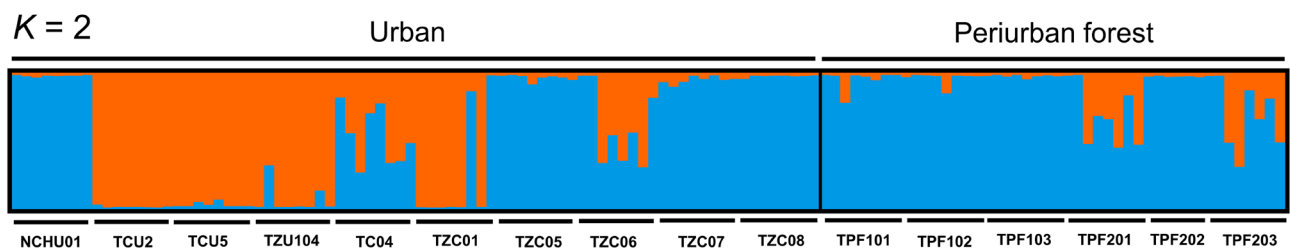


Fig. 4: Population genetic structure estimated through the Bayesian cluster analysis of *Pheidole megacephala* collected from urban areas and periurban forests in Taichung. Each individual is represented by a vertical bar partitioned into different colored (blue and orange) genetic clusters; the length of each segment was proportional to an individual's probability of being assigned to the corresponding cluster.

To determine the difference in the colony structure between the urban and periurban populations, pairwise relatedness (r) was determined by adopting the max-

imum-likelihood method and using ML-Relate (KALINOWSKI & al. 2006). The two habitats were compared in terms of within-colony genetic relatedness among workers

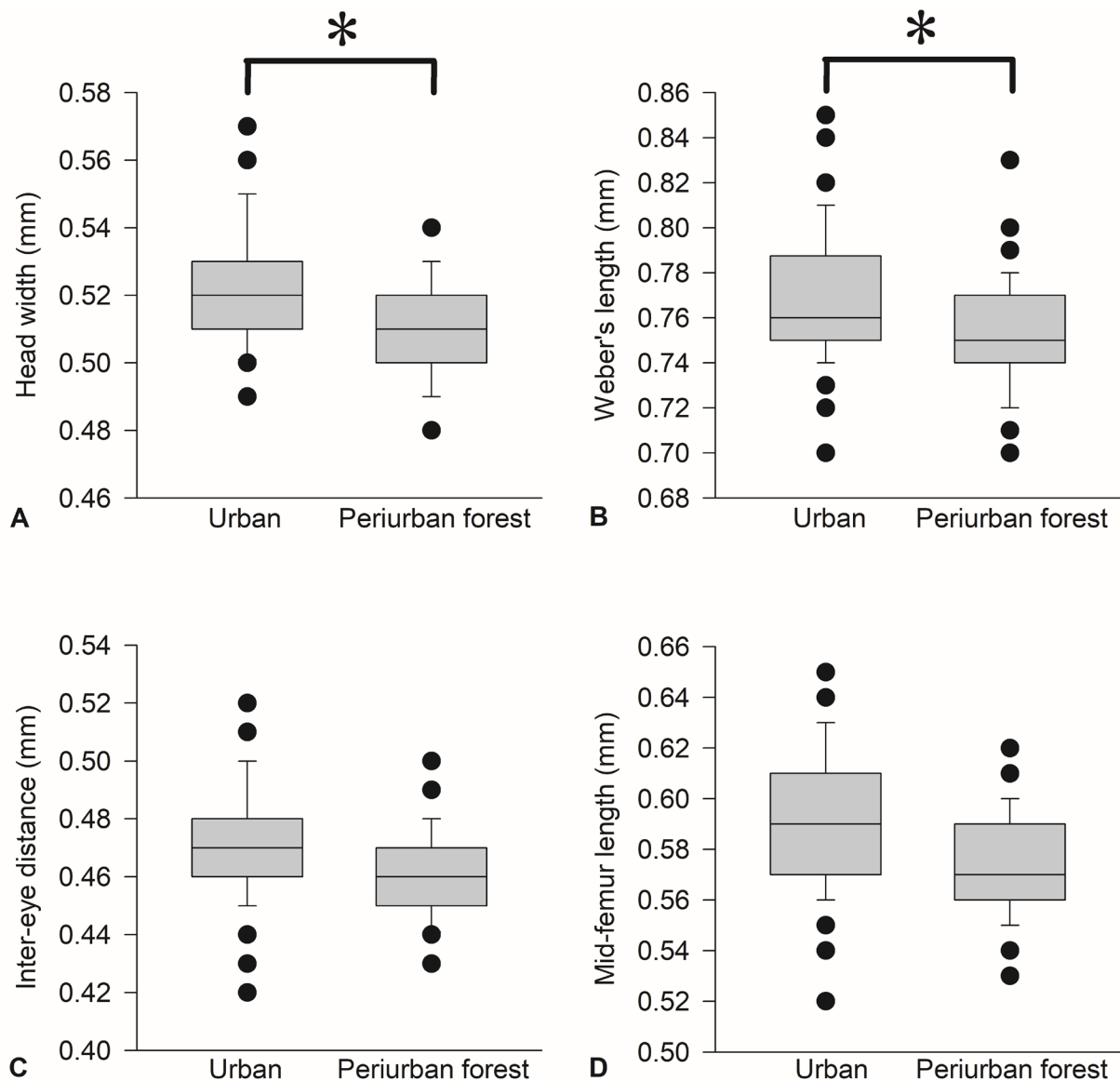


Fig. 5: Distribution of the morphological traits of *Pheidole megacephala* collected from urban areas and periurban forests. Horizontal bars indicate median values, and boxes indicate the 25% and 75% quartiles of the data sets. Asterisks indicate significant difference ($P < 0.05$).

by using an independent Student's t test and in terms of the number of female reproductive individuals in a colony by using the Mann-Whitney U test. Nonparametric tests were performed when the assumption of normality (Shapiro-Wilk test, $P < 0.05$) was not met for the parametric test of data and when data transformation did not improve the normality.

To evaluate the likelihood of gene flow between the urban and periurban forest populations, their population structures were assessed by using the Bayesian-clustering method-based programme STRUCTURE (version 2.3.4) (PRITCHARD & al. 2000). All samples were analysed using an admixture model through a Markov chain Monte Carlo run for 1 million generations with a 100,000 burn-in for a range of possible clusters ($K = 1 - 10$). Each K was tested 10 times. The highest score of K value supported

by Delta K (EVANNO & al. 2005), which was evaluated using the online software Structure Harvester (EARL & VONHOLDT 2012), was selected to generate a graph by using the CLUMPAK server (<http://clumpak.tau.ac.il/>) (JAKOBSSON & ROSENBERG 2007). Hierarchical analysis of molecular variance (AMOVA) (EXCOFFIER & al. 1992) was performed using GenAlEx 6.5 (PEAKALL & SMOUSE 2012) to determine the differences between the samples at three levels (between habitats, between colonies within a habitat, and within colonies). The pairwise comparison fixation index (F_{ST}) for each habitat was evaluated through 999 permutations. Gene flow was estimated using Wright's F -statistics (WRIGHT 1943). Genetic differentiation between the habitats was determined based on the F_{ST} value, and significance was tested by performing a permutation test using GENEPOP (version 4.5) (ROUSSET 2008).

Results

Associations of isotope values with seasons and habitat types

The overall data set analysis performed using the GLMMs indicated that seasons did not exert a significant effect on the mean values of $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ (Tab. 1; Appendix Fig. S1, as digital supplementary material to this article, at the journal's web pages). Regarding habitat types, the mean values of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ were significantly higher in urban areas than in periurban forests. No significant difference was noted in $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ values within habitat type (Tab. 1; Appendix Fig. S1).

Trophic niche breadth

In Taiping, the ants collected from the urban area occupied a larger isotopic space than did those collected from the periurban forest. The Bayesian ellipse areas of the urban and periurban forest habitats in Taiping were 56.6 and 54.5 units of per mil squared, respectively, with an overlapping area of 6.8 units of per mil squared (Fig. 1). Similar to the findings in Taiping, the isotopic space occupied by the urban ants in Tanzi was 21.1 units of per mil squared larger than that occupied by periurban forest ants. The Bayesian ellipse areas of the urban and periurban forest habitats in Tanzi were 60.6 and 39.5 units of per mil squared, respectively, with an overlapping area of 17.2 units of per mil squared (Fig. 1).

Trophic positions

In Taiping, we noted a significant difference in the estimated trophic position of *Pheidole megacephala* between the two habitats ($t = 5.168$, $P < 0.001$, and $df = 65$). The trophic position values for the urban and periurban forest habitats in Taiping were 2.466 ± 0.077 and 1.860 ± 0.078 (mean \pm standard error (SE)), respectively. In Tanzi, the estimated trophic position was significantly higher in the urban area than in the periurban forest (mean \pm SE: urban areas: 2.281 ± 0.082 ; periurban forests: 1.608 ± 0.100) ($t = 4.935$, $P < 0.001$, and $df = 55$; Fig. 2). Notably, the trophic position of the urban populations of *P. megacephala* was comparable with that of a carnivorous species; by contrast, the forest populations of *P. megacephala* occupied a relatively low position that was comparable with that of a herbivorous species (Fig. 2).

Colony structure

N_A per locus was 2 - 6. Six private alleles were discovered; two of them were specific to urban areas and four were specific to periurban forests. As shown in Table 2, no significant difference was observed between the urban and periurban populations in N_A (Mann-Whitney U test, $U = 20.5$ and $P = 0.600$), A_r ($t = -0.053$, $P = 0.958$, and $df = 12$), H_E ($t = 0.938$, $P = 0.367$, and $df = 12$), or H_O ($t = -0.676$, $P = 0.512$, and $df = 12$).

Within-colony genetic relatedness significantly differed from zero-relatedness in both urban ($t = 27.824$, P

< 0.001 , and $df = 279$) and periurban forest ($t = 10.917$, $P < 0.001$, and $df = 154$) habitats. *Pheidole megacephala* workers in urban areas had higher within-colony relatedness than did those in periurban forests (mean \pm SE: urban areas: 0.433 ± 0.016 , periurban forests: 0.194 ± 0.018 ; $t = 10.135$, $P < 0.001$, and $df = 363$; Fig. 3A; Appendix Tab. S1). The genetic relatedness between the colonies within a habitat was low in both urban and periurban forest habitats (mean \pm SE: urban areas: 0.152 ± 0.004 , periurban forests: 0.138 ± 0.007).

The high within-colony genetic relatedness of workers in urban areas is consistent with the small number of female reproductive individuals in these areas. By contrast, periurban forest colonies characterized by low genetic relatedness contained a considerably high number of female reproductive individuals (mean \pm SE: urban areas: 2 ± 1 , periurban forests: 60 ± 25 ; Mann-Whitney U test, $U = 1.5$, and $P < 0.01$; Fig. 3B). The F_{ST} value was higher in urban areas than in periurban forests, revealing considerable differences in the ant colonies sampled from the two habitats ($t = 3.631$, $P < 0.001$, and $df = 58$; Tab. 2). The STRUCTURE analysis revealed that the best value of K was 2, and two genetic clusters were identified, with one cluster including four urban colonies (TCU2, TCU5, TZU104, and TZC01) and the other cluster including the remaining urban colonies and all periurban forest colonies (Fig. 4). The pairwise F_{ST} value was 0.085, which suggested low genetic differentiation between the urban and periurban forest colonies. The gene flow between the urban and periurban forest colonies (N_m) was 2.688. F_{IS} did not differ significantly from 0, indicating that random mating was predominant in both the urban ($t = 1.099$, $P = 0.3$, and $df = 9$) and periurban forest ($t = 1.462$, $P = 0.204$, and $df = 5$) colonies (Tab. 2). The results of AMOVA (Tab. 3) revealed that most of the genetic differentiation could be explained by within-colony differences (69%), followed by between-colony differences within a habitat (25%) and between-habitat differences (6%).

Morphological traits

The GLMMs revealed that the body size of minor workers of *Pheidole megacephala* was larger in urban areas than in periurban forests in Taiping (head width: $t = -2.153$, $P < 0.05$, and $df = 208$; Weber's length: $t = -2.085$, $P < 0.05$, and $df = 208$; Fig. 5). By contrast, the intereye distance and mid-femur length were similar between the colonies of urban areas and periurban forests in Taiping (intereye distance: $t = -0.957$, $P = 0.338$, and $df = 208$; mid-femur length: $t = -1.224$, $P < 0.221$, and $df = 208$; Fig. 5).

Discussion

Carbon and nitrogen isotopic signatures of *Pheidole megacephala* were higher in urban areas than in periurban forests. In addition, ants in urban areas exhibited greater variation in carbon resource intake than ants in periurban forests. These results highlight the trophic niche breadth expansion and the assimilation of high protein resources by *P. megacephala* in an urban environment. By contrast,

exploitation of plant-based resources was apparent in periurban forests. Major reliance on protein-based food may be associated with the colony structure of *P. megacephala* in urban areas which generally exhibited smaller numbers of queens and worker ants; their counterparts in periurban forests which principally consume plant-based resources were characterized by extreme polygyny. The monogyne and low-degree polygyne colonies of *P. megacephala* in urban areas include large-bodied workers.

Diet seasonality

In general, ant colonies depend primarily on protein-based resources for offspring provisioning and on carbohydrate-based resources for reproductive investment before flight activity (JUDD 2005). Nevertheless, evidence for seasonal changes in foraging behavior is limited; inconsistent result has been reported (COOK & al. 2011). In the present study, no significant seasonal variation was noted in $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values. The nonvariance in the isotopic signature might be due to the fact that in both seasons the ants likely consumed different diets with similar isotopic values. Alternatively, food availability in a habitat may have limited the type of food intake despite different nutritional requirement at different colony stages (RICO-GRAY & STERNBERG 1991, COGNI & OLIVEIRA 2004).

Dietary shift in urban areas

An increase in the degree of urbanization-induced disturbance in a habitat may strongly influence the species structure in the habitat. A decrease in keystone-species population causes a shift toward human-food-waste consumption by urban species (MARZLUFF & NEATHERLIN 2006, NEWSOME & al. 2010). In the present study, $\delta^{13}\text{C}$ was higher in the urban populations of *Pheidole megacephala* than in the periurban forest populations. This finding highlights the dietary flexibility of ants; they can shift their carbon intake from C_3 to C_4 sources in urban areas, which suggests a dietary shift toward human fast food, as evident from the overlapping of the Bayesian ellipses in our study. However, the biplot of the carbon and nitrogen isotope ratio indicated that the diet of urban *P. megacephala* ants also included other naturally available food sources.

In this study, unlike ants from Taiping, urban ants from Tanzi exhibited an expanded trophic niche breadth relative to forest ants, as suggested by the Bayesian ellipse areas; this reflects the diversity of diet resources (WALSH & TUCKER 2020). Contrary to the findings of PENICK & al. (2015), our findings revealed that $\delta^{15}\text{N}$ was significantly higher in urban ants than in periurban forest ants in both Taiping and Tanzi, suggesting the elevation of the trophic position of urban ants. Given that *Pheidole megacephala* ants are generalist foragers, we hypothesised that urban *P. megacephala* ants would have adopted a relatively “carnivorous” diet and increased their reliance on a wide range of animal-based resources. By contrast, the current study demonstrated that $\delta^{15}\text{N}$ was significantly lower in the periurban forest populations than in the urban populations, indicating that periurban forest ants are more “herbivo-

rous” than urban ants; that is, periurban forest ants feed on resources at low trophic levels in the food chain. Although *Pheidole* ants are generalist foragers, our findings corroborate those of other studies suggesting that forest *P. megacephala* ants occupy a low trophic position, probably relying on honeydew-producing insects for high-carbohydrate food (WETTERER 2007, PUTRI & al. 2021, BARATELLI & al. 2022). This behavior strongly affects the ability of invasive ants in a competitive new environment to outnumber native ants (KAY & al. 2010). However, the results for ants in Taiping should be interpreted with caution because of the different plant species used for baseline and the large variation in baselines caused by the low number of samples in the study sites.

Colony structure and queen number

The low relatedness between colonies within a habitat and the nonsignificant inbreeding coefficient in periurban forests indicate the likelihood of random mating. A similar dispersal mating strategy was observed in urban areas. However, the mechanism underlying the difference in the degree of polygyny between the urban and periurban forest colonies remains unclear.

Studies comparing the colony structure of invasive ants between urban and natural forests have suggested that colony structure differences are an inheritable trait contributing to invasion success. For example, the polydomous colonies of *Tapinoma sessile* in urban areas are mostly headed by multiple queens, whereas the colonies in natural habitats are typically headed by a single queen (BLUMENFELD & al. 2021). Such a dichotomy between urban and natural populations may result from intense selection pressure (BLUMENFELD & al. 2021). In the present study, gene flow was not completely restricted between the urban and periurban forest populations. Therefore, we postulate that genetic adaptation is not the sole factor shaping the colony structure of *Pheidole megacephala* in urban areas and periurban forests. The simplest explanation may be that changes in resources influence the structure of ant colonies in urban areas.

In general, colony expansion through budding or young queen adoption only occurs when the colony is large and the environment has abundant resources. This is because rearing a female reproductive individual is energetically expensive. In the present study, colony dissection revealed that the urban colonies were mainly monogynous or less polygynous and were headed by a couple of egg-laying queens compared with the periurban forest colonies. This finding is further supported by the significantly higher within-colony genetic relatedness among workers in urban areas than in workers in periurban forests. A potential explanation for the low degree of polygyny in urban colonies is nutritional imbalance. High protein intake is invariably detrimental to colony growth (DUSSUTOUR & SIMPSON 2008, 2012, WILLS & al. 2015). Considerable costs are associated with the dietary shift toward a protein-based insect diet in urban areas. The low within-colony genetic

relatedness among workers in periurban forests indicates that extreme polygynous colonies are headed by a large number of functional female reproductive individuals. The abundance of carbohydrates in the form of nectar or hemipteran-induced honeydew may affect the colony structure and increase the colony size of *Pheidole megacephala*. We previously reported that *P. megacephala* accounted for 31% (781 of 2468 individuals) and 40% (1127 of 2768) of all ants observed in Taiping and Tanzi, respectively, and > 98% of the total ant occurrence at certain transects in the study sites (TSAI 2019).

Morphological traits

Previous work by FOURNIER & al. (2012) with *Pheidole megacephala* reported that head width was smaller in urban *P. megacephala* populations than in forest *P. megacephala* populations in Cameroon. This variation likely resulted from genetic variation attributed to reproductive isolation across a large geographical area (~200 km) (FOURNIER & al. 2012). Urban *P. megacephala* ants are morphologically distinct, with short spines on the propodeum and hairs on the petiole that end in a pointed tip; by contrast, the petiole hairs of forest ants end in a brush-like structure (FOURNIER & al. 2012). We found that gene flow was not restricted between the populations in the two habitats (approximate distance 10 km), although AMOVA revealed that 6% variation could be explained by the between-habitat differences. Furthermore, no cryptic species of *P. megacephala* as described by FOURNIER & al. (2012) has been observed in Taiwan (LIU & al. 2022). Gene flow may be attributed to human-mediated dispersal, which breaks the dispersal barriers (KHIMOUN & al. 2020). Our findings corroborate those of WILLS & al. (2014), who observed no significant involvement of phylogenetic traits in the body size variation and caste ratio of five *P. megacephala* populations collected from Hawaii, Mauritius, Florida, Australia, and South Africa. By contrast, our findings suggest that any shifts in body size traits were driven by feeding plasticity in response to urbanization rather than by genetic variability.

Previous studies suggest that urban ants had a body size smaller than that of forest ants due to the major reliance on protein-based food (DUSSUTOUR & SIMPSON 2008, WILLS & al. 2015). However, we obtained opposite findings. The head width and Weber's length of urban ants were significantly larger than those of periurban ants. The macronutrient composition is unlikely to explain the increased body size; nonetheless, this result may be associated with polygyny in ants. Several empirical studies have revealed the increased worker size in the monogynous colonies of *Solenopsis invicta* (GREENBERG & al. 1985, PORTER 1992, GOODISMAN & al. 1999), which may be because monogynous colonies with a low brood load have a higher proportion of workers for brooding than do polygynous colonies. Hence, the brood in monogynous colonies receives more care and nutrients than does the brood in polygynous colonies (PISARSKI 1981).

Foraging in the urban environment may be risky for ants; it may be regarded as a key factor influencing colony survival. The shift toward a large body size in urban areas may reflect the phenotypic plasticity driven by urbanization: A large body size maximizes the chance of survival in the urban environment. Consistent with this notion, larger *Pheidole megacephala* ants may have a higher level of tolerance to environmental stress, such as the low humidity in urban areas; similar observations have been reported for other taxa (HU & al. 2012, BONG & al. 2018) and metal pollution (VESELA & VIJVERBERG 2007, GRZEŚ 2010, JACQUIER & al. 2021). Moreover, individuals with a larger body size exhibit a higher level of heat tolerance (RIBEIRO & al. 2012, BAUDIER & O'DONNELL 2018); thus, larger individuals have more advantages in foraging under the UHI effect (CLÉMENCET & al. 2010). An alternative hypothesis is that larger ants would be selected for survival in resource-depleted urban environments because these individuals possess higher levels of energy for foraging and exhibit higher levels of endurance during starvation under harsh environments (GERGS & JAGER 2014). Therefore, a large body size is crucial for urban *P. megacephala* ants because it facilitates foraging over a large distance in a patchy resource habitat for an extended period.

Conclusions

The trophic niche breadth of *Pheidole megacephala* was comparable or larger in urban areas when compared with forest areas. However, we noted an elevated trophic position similar to that of a carnivore in urban ants. Together, these findings suggest the dietary dependence of this species on high-protein resources, which might have resulted in the small numbers of reproductive individuals and workers in urban areas. The monogynous and low-degree polygynous colonies of *P. megacephala* in urban areas include large-bodied workers despite a high imbalance in protein intake. A large body size appears to be a key functional trait of urban *P. megacephala* ants in the context of survival and efficient foraging.

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