



Climate microrefugia promote intraspecific trait variability of the Palaearctic ant species *Myrmica ruginodis* (Hymenoptera: Formicidae)

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Abstract

Topographic depressions in karst areas (dolines) play an outstanding role in maintaining small-scale environmental heterogeneity in homogeneous landscapes. Such depressions can serve as microrefugia for cool-adapted species, providing cooler and more humid conditions than the surrounding matrix (plateaus). Recent studies showed that dolines can maintain species with different functional traits compared with the surrounding areas, providing a more heterogeneous and competitive environment. However, we have limited knowledge pertaining to how microrefugia shape the functional traits of a single species. Here, we compared the functional traits of the Palaearctic ant species *Myrmica ruginodis* between microhabitats of dolines and the surrounding plateaus in a karst landscape. We found that the number of virgin queens was higher on the plateaus, while males were more numerous in dolines, but there was no significant difference in colony size between the two microhabitats. Dolines hosted more worker brood, suggesting potential for larger colonies. Interestingly, the repeatability of worker aggressiveness was higher and worker size was more variable in dolines, emphasizing the individual aspects of doline colonies. Our results highlight that dolines not only support species whose functional traits vary from those occurring on the surrounding plateaus, but these microhabitats also exert a filter effect on the functional traits of a given species, enriching our understanding of biodiversity maintenance. It seems that – although the biotic and abiotic conditions provided by these microrefugia are more favorable for *M. ruginodis* than the surrounding plateaus – the plasticity of their functional traits allows them to compensate and survive even in sub-optimal environmental conditions. This plasticity may prove to be a useful prospect in the light of the ongoing climate change.

Key words: Karst dolines, functional traits, behavioural variability, aggressiveness, interspecific competition.

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Introduction

Topographically complex landscapes can create habitats with unique microclimates that are decoupled from regional climates (DOBROWSKI 2011, OLDFATHER & al. 2020). Such habitats may function as climate microrefugia that support species persistence during climatic changes (RULL 2009, LENOIR & al. 2017). For instance, ravines and valleys promote cold-air pooling that sustains temperature inversions and low temperatures but also determines other key environmental factors, such as relative air humidity, soil moisture, and soil temperature (PASTORE & al. 2022). Identifying and understanding climate microrefugia is crucial for conservation planning and management (KEPPEL & al. 2015, MORELLI & al. 2017). Long-term temperature measurements, species distribution modelling, genetics, and the contemporary distribution of relict species and populations may enable researchers to describe complex processes that create long-term safe havens for biodiversity (KEPPEL & al. 2012). More recently, it has been highlighted that eco-evolutionary selective forces may influence the functional signature of species assemblages in microrefugia, resulting in distinct values of functional traits, functionally similar traits belonging to distantly related lineages, and greater functional diversity (KEPPEL & al. 2018, OTTAVIANI & al. 2019). Nonetheless, our knowledge remains limited concerning how microrefugial processes shape intraspecific functional trait variability within a landscape.

Functional traits are morphological, biochemical, physiological, or behavioural characteristics that play an important role in the fitness performance of species (HOOPER & al. 2005, MCGILL & al. 2006, NOCK & al. 2016). Response functional traits are particularly important in determining the resilience of species and tolerance to disturbances as they determine how a species can respond to changes in environmental conditions (DÍAZ & al. 2013). Previous studies have shown that many species have the potential to cope with changes in their environment by varying their functional traits (AHRENS & al. 2019, ILYAS & al. 2021), highlighting that a wide range of habitat conditions, such as air temperature and relative air humidity, significantly influence specific functional traits (LEBRIJA-TREJOS & al. 2010, ARNAN & al. 2014, GARCÍA-LLAMAS & al. 2019, NOOTEN & al. 2019, DEÁK & al. 2021). For instance, in south-western Mediterranean ant communities, the number of queens and nests per colony increased significantly with precipitation seasonality, whereas the average worker size and worker polymorphism increased with temperature seasonality (ARNAN & al. 2014). The diversity and availability of food sources are another important factor that may shape ant functional traits (REYMOND & al. 2014). When resource diversity is limited, the number of foraging strategies is also reduced. Conversely, a greater diversity of food sources leads to a wider range of foraging strategies (REYMOND & al. 2013, ARNAN & al. 2014). Previous studies have also shown that vegetation can affect the colony size of *Myrmica* ants. Colonies from habitats dominated by invasive plants were

smaller compared with those from control areas (LEDA & al. 2013, GRZÉS & al. 2018). Furthermore, GRZÉS & al. (2018) found that *Myrmica* colonies from invaded habitats maintain more gynes than those from non-invaded habitats. In general, queens from monogynous colonies (with a single queen) are characterized by large body size, good dispersion, and colony-founding ability, making them more successful in colonizing favorable areas that are further away from each other or with less favorable conditions (KELLER 1995). Additionally, the number of queens within a colony can influence other functional characteristics, such as the number of workers, their size, and competitive ability, thereby significantly affecting the fitness of the colony (REYMOND & al. 2013).

In this study, we examine the trait distribution patterns of a Palaearctic ant species in microhabitats inside and outside topographic depressions (dolines) in a mountainous karst landscape in Northern Hungary. Dolines, formed by dissolution processes, are typically circular in plan form (FORD & WILLIAMS 2007). Due to the thermal inversion, dolines maintain cooler and moister conditions, potentially serving as important microrefugia for biodiversity (BÁTORI & al. 2022, FREI & al. 2023). For example, compared with the surrounding plateaus, dolines can support species associated with moist habitats, but also small-bodied spiders, carnivorous ground beetles, and rove beetles with high dispersal capacity (BÁTORI & al. 2022). Similarly, in ants, dolines had a significantly higher number of species adapted to cooler and moister conditions that were moderately aggressive compared with the plateaus (BÁTORI & al. 2020). This suggests that dolines may facilitate the persistence of species with specific traits that are otherwise eliminated from the surrounding landscape (BÁTORI & al. 2022).

We selected ants as model organisms because they are one of the most abundant and diverse insects worldwide (HÖLLDOBLER & WILSON 1990). Moreover, ants are reliable indicators of small-scale environmental heterogeneity and are especially sensitive to changes in their environment (SANDERS 2002, ANDERSEN & MAJER 2004, ARNAN & al. 2014, TIEDE & al. 2017, JUHÁSZ & al. 2020). Consequently, the environment inhabited by ants can select for their functional traits, illustrating the complex relationships between trait diversity and various environmental factors (DOLEK & al. 2009, REYMOND & al. 2013, ARNAN & al. 2014, DEÁK & al. 2021). Our model species, *Myrmica ruginodis* NYLANDER, 1846, is widespread throughout the northern Palearctic, ranging from Western Europe to Japan. It prefers cool and humid environments, typically nesting in forests, shrubs, and bogs (CZECHOWSKI & al. 2012). This preference explains why *M. ruginodis* is commonly found on north-facing doline slopes and is much rarer on the surrounding plateaus (in Northern Hungary), which can be up to 15 °C warmer during the daytime than the north-facing doline slopes (BÁTORI & al. 2019, 2020, 2023).

We hypothesize that the presence of doline microrefugia significantly influences the functional traits of *Myrmica ruginodis* at both the individual and colony level.

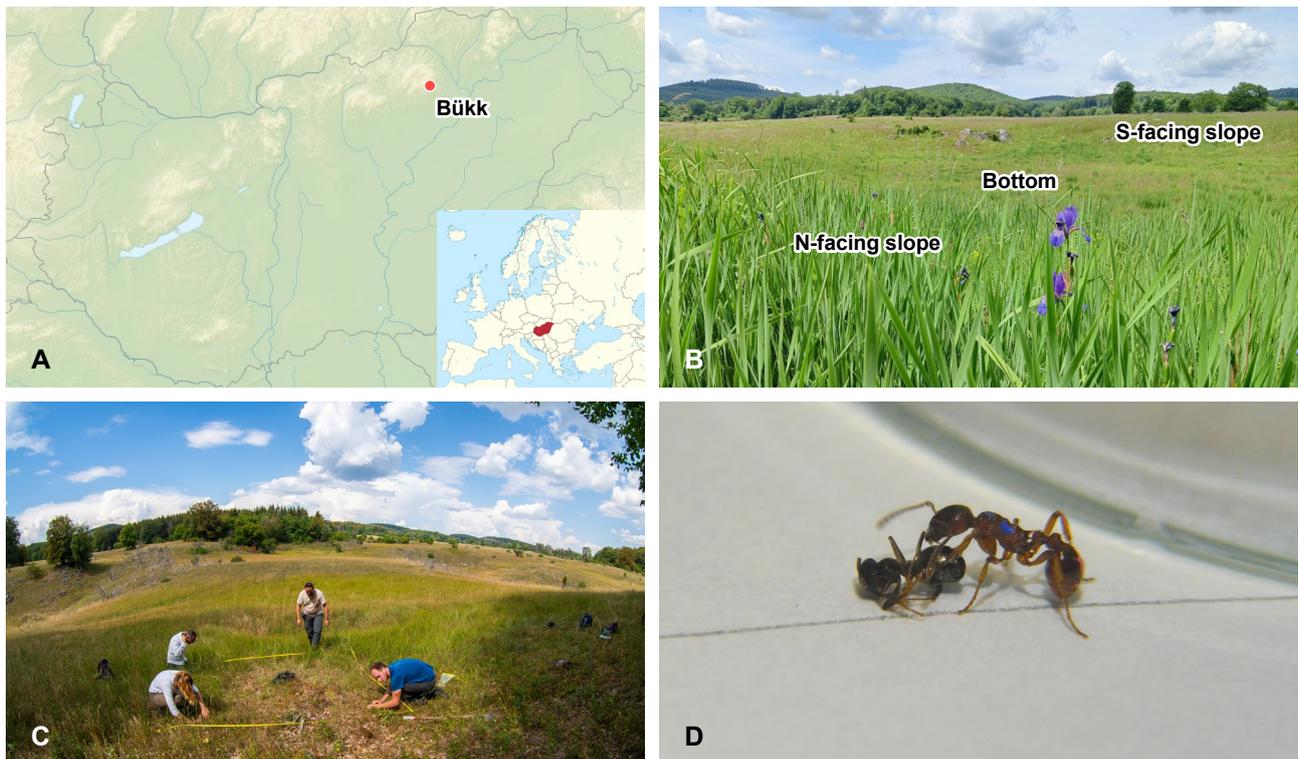


Fig. 1: Location of the study site in the Bükk Mountains in Northern Hungary (A); a grassland doline with different microhabitats (B; photo: Zoltán Bátori); assessment of nest density within a 3 m × 3 m quadrat on the plateau (C; photo: Gábor Li); confrontation of a *Myrmica ruginodis* worker with a corpse of *Lasius niger* in the aggression assay (D; photo: Fanni Pécsy).

Dolines may provide important microhabitats for many ant species and other taxa associated with cool and / or moist conditions, and their funnel-shaped topography can trap a high number of good dispersers (BÁTORI & al. 2020, 2022). Consequently, we expect that interspecific competition for space and resources, primarily leading to a lack of protein, is more intense in dolines than on the surrounding plateaus, potentially leading to lower fitness in these habitats. Moreover, populations coming from habitats with different environmental characteristics may exhibit differences in behavioural traits, including individual behavioural expression and repeatability (VAN DONGEN & al. 2010, OLDHAM & al. 2019, MAÁK & al. 2021). Given that larger colonies with variable worker sizes can confer competitive advantages (HÖLDOBLER & WILSON 1990, CERDÁ & al. 2009, RUEL & al. 2012), we expect that colonies of *M. ruginodis* in dolines will be larger and exhibit greater worker size variation to successfully mitigate the negative effects of competition. Furthermore, we also expect that workers from dolines will display higher levels of aggressiveness compared with those from plateaus, along with greater repeatability of aggression, as individual strategies may confer greater benefits in these environments.

Material and methods

Study area

Our study was carried out in a karst landscape of the Bükk Mountains in Northern Hungary (48° 04' 35.4" N,

20° 29' 34.5" E; Fig. 1 A), where dolines occur in large numbers (BÁRÁNY-KEVEI 1999). The mean annual temperature is 6.3 °C, and the mean annual precipitation is 800 mm (DÖVÉNYI 2010). The altitude ranges between 770 and 870 m above sea level.

There are significant variations in microclimates among doline microhabitats and the surrounding plateaus. For instance, BÁTORI & al. (2019) found mean daily temperatures to be more than 5.5 °C warmer on south-facing than on north-facing doline slopes and more than 3.5 °C warmer on the plateaus than on north-facing doline slopes (in August 2017). Mean relative humidity was 10% higher on north-facing than on south-facing slopes and 8% higher on north-facing slopes than on the plateaus. Microclimatic studies over longer periods (from a few days to a year) also demonstrated that north-facing slopes and bottoms of dolines are consistently colder and more humid than the surrounding microhabitats (WAGNER 1963, BÁRÁNY-KEVEI 1999, MARCIN & al. 2021), such as the plateaus. Furthermore, dolines in the Bükk Mountains play an important role in maintaining high vegetation diversity. BÁTORI & al. (2023) found that variations in vegetation within dolines are influenced by different environmental gradients, such as temperature, humidity, and soil pH, resulting in distinct vegetation cover on the various slopes. Additionally, due to conditions different from the plateaus, dolines can support plant species that are absent from the surrounding areas (BÁTORI & al. 2023). The dominant tree species within the study area is *Fagus sylvatica*, but there are also patches

of *Picea abies*. Diverse grassland communities also cover large areas, consisting of many rare, endangered, and protected plant species (BÁTORI & al. 2023). The cool and humid north-facing doline slopes and doline bottoms are covered by hay and / or wet meadows that have the capacity to maintain the populations of mountain and relict species, such as *Alchemilla monticola* or *Bupleurum longifolium*, while the warm and dry south-facing doline slopes are covered by dry, rocky grasslands with species such as *Anemonoides sylvestris* and *Iris variegata*. The plateaus are covered by semi-dry grasslands (VOJTKÓ 2001, BÁTORI & al. 2023). The grassland dolines of the Bükk Mountains also harbour a high diversity of ant species with different abiotic preferences. BÁTORI & al. (2020) found a total of 22 ant species, with species like *Myrmica ruginodis* and *Myrmica sabuleti* as diagnostic species for dolines, while *Lasius niger* and *Myrmica scabrinodis* were more typical for the plateaus.

Model species

Our model species was the ant *Myrmica ruginodis* NYLANDER, 1846, which is a mesohygrophilic species widespread in Western-, Central-, Eastern- and Northern-Europe, Siberia, the Far East, but also Japan (RADCHENKO & ELMES 2010, CZECHOWSKI & al. 2012). Their colonies can be found mostly in cooler and moist habitats like forests, shrubs, bogs, and alpine meadows, but they avoid open habitats with highly wet or dry conditions (RADCHENKO & ELMES 2010). *Myrmica ruginodis* workers are generalist predators, their food consists mainly of small invertebrates, but they also consume honeydew produced by aphids (CZECHOWSKI & al. 2012). Colonies are monogynous (one queen) or polygynous (multiple queens). Queens in monogynous colonies are larger than queens in polygynous colonies. As these smaller queens have a better dispersal ability, they are more successful in colonizing new, more isolated habitats and can establish colonies even in less favourable conditions (RADCHENKO & ELMES 2010, CZECHOWSKI & al. 2012). The mating flight period lasts from August to September, but in colonies living in the mountains, this can even be postponed until mid-October (RADCHENKO & ELMES 2010, CZECHOWSKI & al. 2012).

Field and laboratory data collection

Five sampling sites were established within the study area in August 2022. Each site consisted of one doline and one plateau location (5 sites \times 2 locations: 10 locations in total; Fig. 1 B). Two *Myrmica ruginodis* colonies were chosen from the north-facing slope of each doline and the related plateau location (Fig. 1 B), respectively (5 sites \times 2 locations \times 2 colonies: 20 colonies in total). The two neighbouring colonies were at least 15 m apart to avoid the overlapping of their home ranges. The nest density of all ant species was thoroughly assessed around each *M. ruginodis* colony in a 3 m \times 3 m quadrat (in the middle of which was the focal *M. ruginodis* colony) by investigating all potential nesting sites in the soil surface (Fig. 1 C).

Focal colonies and a relatively large area around each of them were excavated, ensuring that no more ants were present in the surrounding area (such procedure was used in all nest dimensions). The field-collected colonies were transported to the laboratory of the Ecology Department of the University of Szeged, Hungary. Ants were identified to species or genus level in the field, with at least three representatives collected and preserved in 95% ethanol for subsequent species identification in the laboratory via the keys of CZECHOWSKI & al. (2012) and SEIFERT (2018). The specimens were deposited at the Department of Ecology, University of Szeged.

The functional traits of each collected colony were determined (i.e., colony parameters) in the laboratory by meticulously dismantling their nests and recording all colony members found. These were the number of workers, mature queens, virgin queens (gynes), males, larvae, and pupae. Following this, the colonies were housed with the collected nest material in a transparent plastic box (19 cm \times 19 cm \times 10 cm) under identical laboratory conditions (temperature $22 \pm 4^\circ\text{C}$; relative humidity 42 - 43%; 12:12 h light:dark cycle). The walls of nest boxes were coated with paraffin to prevent ants from escaping. A small piece of wet sponge was added to each colony to maintain appropriate humidity in the nest boxes. Ants were fed twice per week with a 1:2 honey-water mixture and tuna chunks as the protein source. All colonies were provided with a specific amount of food relative to their colony size.

Aggression assays

The main goal was to evaluate aggression within the animal personality framework, also referred to as “behavioural consistency” (cf. DINGEMANSE & WOLF 2010, CARTER & al. 2013). Specifically, the aim was to test whether there is evolutionary / ecological among- and within-individual variation in aggressive behaviour between doline and plateau populations. To test this, 14 *Myrmica ruginodis* workers per colony were individually paintmarked using a unique colour combination on the thorax and abdomen with the help of Edding (Ahrensburg, Germany) enamel paint markers. Every marked worker was confronted with a freshly defrosted corpse of *Lasius niger*, a potential competitor species of *M. ruginodis* (Fig. 1 D). *Lasius niger* specimens were euthanized by cooling and subsequently frozen at -20°C and were thawed 10 min before the experiments. A new corpse was used for each *M. ruginodis* worker tested. During the assays, the *M. ruginodis* worker to be tested was placed under a Petri dish, and after 1 min acclimatization, the fresh *L. niger* corpse was also placed under the Petri dish. The first reaction of the *M. ruginodis* worker to the corpse was observed during the assays. The Petri dish was replaced with a new one (previously washed with alcohol) after each test to avoid the potential confounding effects of chemical traces left behind by living or dead ants. The initial encounter, which was defined as the first interaction between the focal ant and the corpse, was recorded and scored as follows: fleeing (rapid movement of the focal ant in the opposite direction) = 1; antennation = 2;

mandible opening = 3; and biting, stinging or carrying = 4. In every individual, the aggression assay was repeated three times, with at least 48 h among consecutive tests. Behavioural assessments were conducted blind to the test animals' identity; only one coordinator, who was not involved in the behavioural analyses, was aware of their identity.

Worker size

To measure their body size, 20 workers for each colony were randomly selected. Ants were stored in alcohol until their measurement. The maximum head width was chosen as a proxy for body size (DESLIPPE & SAVOLAINEN 1994, HAATANEN & SORVARI 2013). For the measurements, a Nikon (Tokyo, Japan) SMZ745T Stereo Microscope was used with 5× magnification. A Nikon Digital Sight DS-fi2 Microscope Camera was used to connect the microscope to the computer, and the head widths were measured using the imaging software NIS-Elements BR 4.13.04 64bit edition. The image size was 640 × 480 pixels, and 0.01 mm / pixel setting was used.

Ethical note

The stress affecting the ants during their collection and transport was minimized as much as possible. Colonies in the laboratory were maintained under nearly natural living conditions, thereby maximizing their welfare and survival. Only behavioural observations and non-invasive contact with the ants were performed during the assays. Except for the individuals used as proxies in the aggression assays or for their head size measurements, the workers were not harmed intentionally or subjected to stressful situations. After the end of the assays, colonies were kept in the laboratory until their natural death.

Statistical analysis

Permutational multivariate analysis of variance (PERMANOVA) was used based on Bray-Curtis dissimilarity and 999 permutations to reveal differences in the colony parameters of *Myrmica ruginodis* between the doline and plateau microhabitats. Because mature queens were not observed in some colonies, the number of queens was excluded from this analysis to avoid a reduction in sample size. Non-metric multidimensional scaling (NMDS) with Bray-Curtis dissimilarity was used to visualize differences in colony parameters (LEGENDRE & LEGENDRE 1998, OKSANEN 2011).

Ant nest densities registered in the quadrates around our focal *Myrmica ruginodis* colonies were compared between the doline and plateau microhabitats using a generalized linear mixed-effects model (GLMM, Poisson error, maximum likelihood fit). The effect of habitat type on the number of *M. ruginodis* workers and queens (only queenright colonies were included) was tested with GLMMs (Poisson error, maximum-likelihood fit). Separate GLMMs (binomial error, maximum-likelihood fit) were built to test the effect of habitat type on the number of larvae, pupae, gynes, and males per worker, where the

respective progeny number weighted by the number of workers was used as a dependent variable. Mean head size – as a proxy of worker body size – was compared with a linear mixed-effects model (LMM, maximum-likelihood fit) between the doline and plateau microhabitats. In the mixed-effects model (LMM), habitat type was included as an explanatory factor, while site and colony IDs were random factors. Variance in head size was compared between the two habitat types with a variance ratio test.

A repeatability estimate was carried out from the data that basically summarizes the amount of variation in behaviour attributable to differences among individuals and used as a proxy for the presence / absence / strength of animal personality. The higher the repeatability estimates, the stronger the individual differences within the population (cf. ROYAUTÉ & DOCHTERMANN 2021). To estimate repeatability in aggression across different habitats, a double hierarchical mixed modelling (DHMM) approach was applied. In this approach, the “mean model” estimates whether individuals differ in their mean expression of behaviour, while the “residual model” estimates whether they differ in residual intra-individual variation around this behavioural mean. As aggression was estimated as a discrete ordinal variable with values varying between 1 and 4, the data were analysed by fitting an ordinal regression DHMM (see BÜRKNER & VUORRE 2019). To assess the potential effect of habitat on behavioural type, a population intercept to the mean model was added (β_0 ; i.e., threshold in an ordinal model), with the fixed effect “microhabitat” (doline vs. plateau). Furthermore, a probit link function was specified to the model to assume the latent variable (equivalently, the error term ϵ) being normally distributed. In our residual model, the parameter related to latent variances is called disc. It is important to note that disc is not the variance itself, but the inverse of the standard deviation (ω). In addition, as the disc parameter must be strictly a positive value, it is by default modelled on the log scale (BÜRKNER & VUORRE 2019). To directly estimate ω , a “non-linear formula” command was used in our residual model (that is $\text{disc} \sim 1 / \exp(\omega)$; see detailed code in Code S1 in the digital supplementary material to this article, at the journal's web pages), also, an identity link function was fitted. To retain the mean disc being 1, no intercept (γ_0) was set to the residual model, only “habitat” as a sole fixed effect.

Adjusted repeatability estimates were calculated from our DHMM by dividing the among-individual level variance by the total phenotypic variance.

$$R = V_{\text{individual}} / (V_{\text{individual}} + V_{\text{residual}})$$

Four chains were run to evaluate convergence, which was run for 5000 iterations, with a warmup of 1000 iterations and a thinning interval of 4. All estimated model coefficients and credibility intervals were therefore based on 4000 posterior samples and had satisfactory convergence diagnostics with R-hat (potential scale-reduction factor on split chains) < 1.01, and effective sample sizes

>1000 (VEHTARI & al. 2020). The mean and 95% credibility intervals are reported, calculated as the highest posterior density intervals, for all parameters in the statistical models to assess whether the parameters were statistically different from 0.

All statistical analyses were carried out with the program R (R CORE TEAM 2022). All results with $p < 0.05$ were considered significant. PERMANOVA was performed using the “adonis2” function, while NMDS was performed using the “metaMDS” function in the vegan package (OKSANEN & al. 2020). GLMMs and LMM were performed using the “glmer” and “glmer.nb” functions in the lme4 package (BATES & al. 2015). In overdispersed models, a negative binomial error structure was applied (see LINDÉN & MÄNTYNIEMI 2011). The variance in head size was computed with the “var.test” function in the stats package. Double hierarchical mixed modelling (DHMM) approach was performed using the R package brms (BÜRKNER 2017, 2018) based on the Bayesian software Stan (version 2.26, STAN DEVELOPMENT TEAM 2021).

Results

Ant nest densities around the focal colonies

We found 47 nests belonging to six species (*Lasius distinguendus*, *Lasius platythorax*, *Leptothorax acervorum*, *Myrmica lobicornis*, *Myrmica ruginodis*, *Myrmica scabrinodis*) around the focal *M. ruginodis* colonies in dolines (0.52 ± 0.32 , mean nest density / $m^2 \pm$ standard deviation SD) and 26 nests belonging to four ant species (*Lasius niger*, *L. platythorax*, *M. ruginodis*, *M. scabrinodis*) around the focal *M. ruginodis* colonies on the plateaus (0.29 ± 0.14 , mean nest density / $m^2 \pm$ SD). Nest density in dolines was significantly higher than on the plateaus (GLMM $z = 2.44$, $p = 0.015$; Fig. 2).

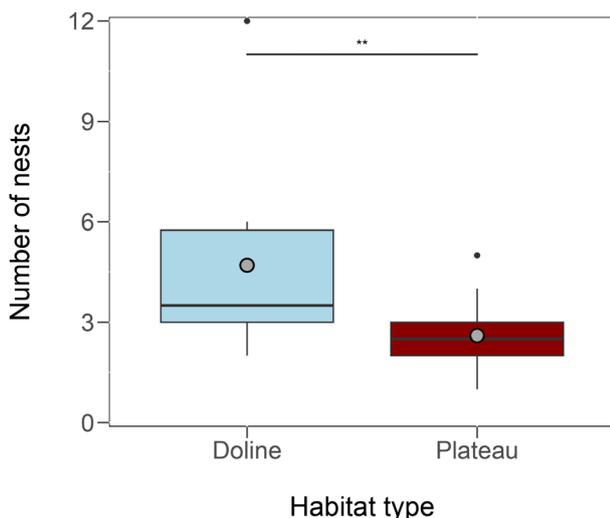


Fig. 2: Differences in the number of nests of all ant species between the two microhabitats (dolines - blue, plateaus - red) that were assessed around each *Myrmica ruginodis* colony within a $3\text{ m} \times 3\text{ m}$ quadrat. Means (grey circle), medians, quartiles, min-max values, and outliers are shown (**: $p = 0.01$).

Effect of habitat type on the functional traits of the focal colonies

According to PERMANOVA, overall colony parameters were not significantly different between doline and plateau microhabitats ($F = 1.03$, $R^2 = 0.05$, $p = 0.355$; Fig. 3). The number of mature queens was significantly higher on the plateaus ($z = 2.09$, $p = 0.036$), whereas there was no significant difference in the number of workers between the two microhabitats ($z = 1.35$, $p = 0.177$; Fig. 4 A). The number of larvae per worker ($z = 7.47$, $p < 0.001$; Fig. 4 B) and males per worker ($z = 2.62$, $p = 0.009$; Fig. 4 C) were significantly higher in the dolines than on the plateaus. Similarly, the combined number of larvae and pupae ($z = 6.84$, $p < 0.001$) was also higher in the dolines, suggesting that a significant portion of the workers was still in the brood stage at the time of collection. Contrarily, the number of pupae per worker ($z = 2.43$, $p = 0.015$) and the number of gynes per worker ($z = 13.44$, $p < 0.001$; Fig. 4 D) were significantly higher on the plateaus.

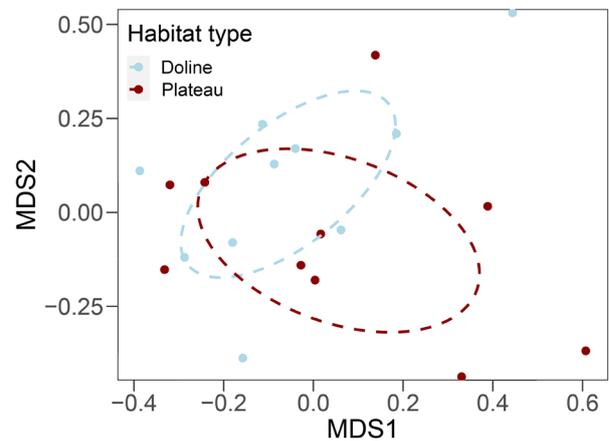


Fig. 3: Visual representation of the non-metric multidimensional scaling for the colony parameters of *Myrmica ruginodis* for the doline and plateau microhabitats (stress value = 0.113). Blue (doline) and red (plateau) circles indicate the location of colonies in the ordination space, while covariance ellipses illustrate the separation of the colonies.

Effect of habitat type on worker size and aggressiveness

The mean head size of the workers did not differ significantly between the doline [8.09 (8.04 - 8.14), mean (95% credibility interval CI)] and the plateau [8.03 (7.97 - 8.09), mean (95% CI)] microhabitats ($t = -0.43$, $N = 400$, $p = 0.670$; Fig. 5). However, the variance in head size was larger in doline [0.19 (0.16 - 0.23), variance (95% CI)] compared with the plateau [0.13 (0.11 - 0.16), variance (95% CI)] colonies ($F = 0.68$, $N = 400$, $p = 0.007$; Fig. 5).

Repeatability estimates from our DHMM indicated that consistent individual differences in aggression (i.e., animal personality) were present in both doline and plateau populations: mean = 0.47 (95% CI = 0.36 - 0.58) vs. 0.21 (0.09 - 0.35), respectively (Fig. 6). Repeatability in

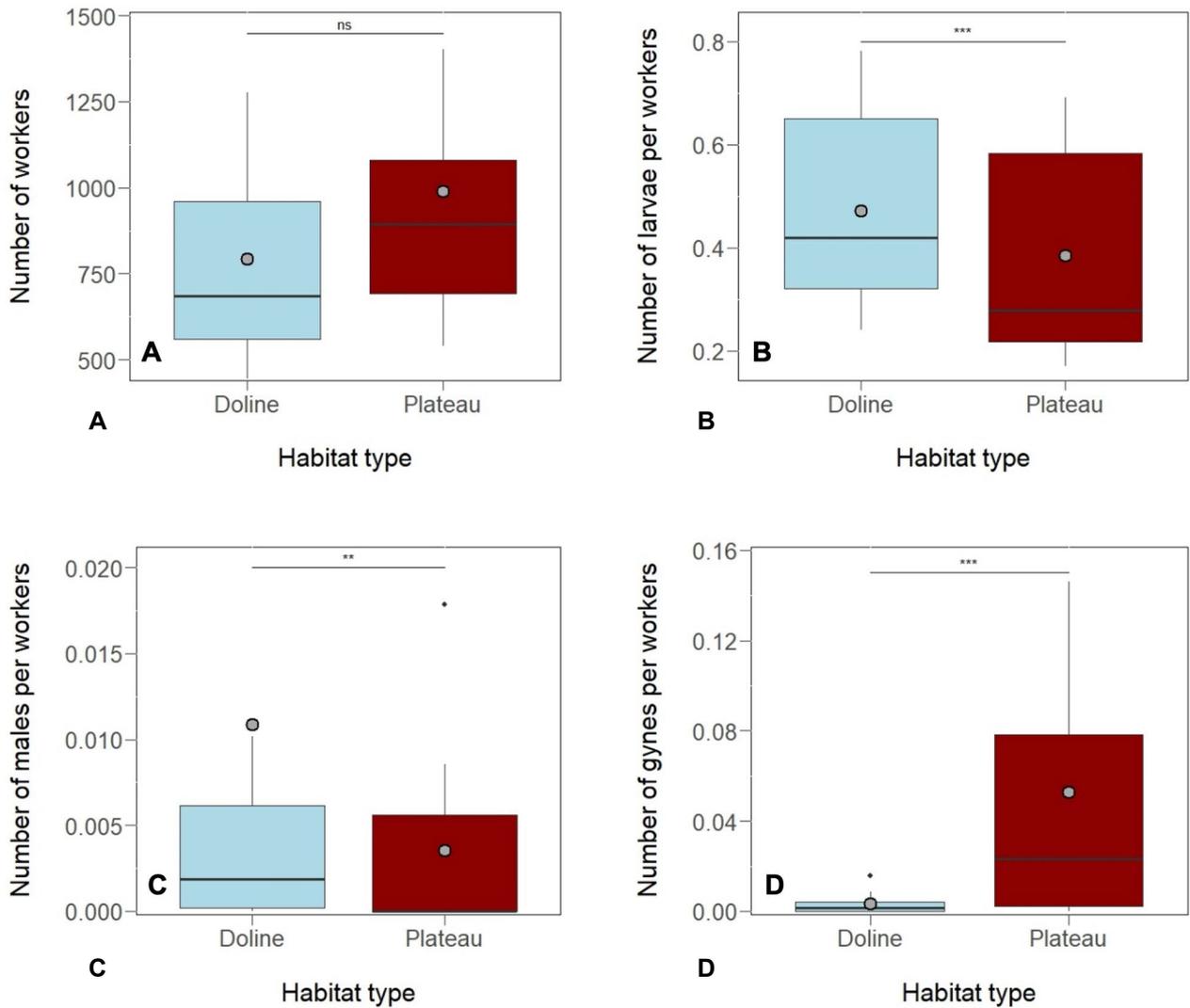


Fig. 4: Differences in the number of *Myrmica ruginodis* (A) workers, (B) larvae, (C) males, and (D) gynes between the doline and plateau microhabitats. Means (grey circle), medians, quartiles, min-max values, and outliers are shown. The number of larvae, males, and gynes were weighted with the number of workers (***: $p \leq 0.001$, **: $p < 0.01$, ns: $p > 0.05$).

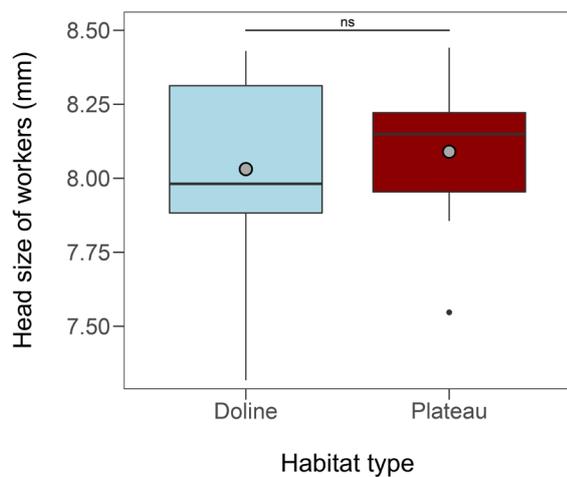


Fig. 5: Difference in head size of *Myrmica ruginodis* workers between the doline and plateau microhabitats. Means (grey circles), medians, quartiles, min-max values and outliers are shown (ns: $p > 0.05$).

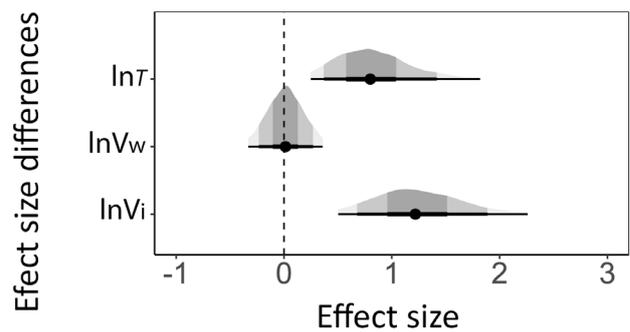


Fig. 6: Effect size differences in repeatability (τ), between- and within-individual variation (V_i and V_w , respectively) of aggression of *Myrmica ruginodis* between doline and plateau microhabitats. Log variance ratios ($\ln VR$) are shown. Whiskers of different thickness represent the 50%, 85%, and 95% credibility intervals.

Tab. 1: Between- (V_i) and within-individual variance (V_w) and repeatability (τ) estimates of aggressiveness of *Myrmica ruginodis* in doline and plateau microhabitats. Means and 95% credibility intervals (CI) are shown. Significant differences are bolded.

Variance and ratio	Environment		Difference
	Doline median (95% CI)	Plateau median (95% CI)	Δ (Doline – Plateau) median (95% CI)
V_i	25.53 (2.13 – 59.84)	7.72 (0.13 – 20.11)	17.81 (0.78 – 42.59)
V_w	29.21 (2.29 – 69.79)	28.74 (2.14 – 69.14)	0.47 (-11.88 – 13.07)
τ	0.47 (0.36 – 0.58)	0.21 (0.09 – 0.35)	0.26 (0.08 – 0.42)

dolines was found to be significantly higher than that of plateaus, as well as between-individual behavioural variation (Tab. 1, Fig. 6).

Discussion

To the best of our knowledge, this is the first study to demonstrate how contemporary and potential future climate microrefugia may promote intraspecific trait variability of arthropod species, highlighting their functional trait plasticity that can allow their survival in habitats with different environmental conditions. We found that the presence of doline microrefugia contributed to significant productivity and structural differences in ant colonies compared with the surrounding plateaus. Additionally, workers in doline colonies exhibited a stronger expression of individual aggressive strategies, which were underpinned by the higher worker size variance observed in dolines. However, mean colony and worker sizes did not differ between the plateau and doline microhabitats, although the potential for larger colonies is present in dolines due to the higher number of broods per worker. The higher ant nest density in dolines, resulting in increased competition and decreased resource availability, is also reflected by the number of gynes, which require more protein than workers and males. Thus, we conclude that the functional trait differences mirror the variations in environmental factors between the plateau and doline microhabitats, leading to overall similar outcomes among populations.

Previous studies have shown that climate microrefugia, such as dolines, can play an important role in maintaining environmental heterogeneity within karst landscapes (e.g., MARCIN & al. 2021, BÁTORI & al. 2022). Consequently, dolines and their surroundings not only host species assemblages with various functional characteristics (BÁTORI & al. 2019, 2020, 2022), but also populations of a single species with different functional traits, such as *Myrmica ruginodis* in the present study. Our results showed that ant nest density was higher in dolines than on the surrounding plateaus. As these microhabitats have the potential to provide microhabitats for diverse ant assemblages and collect arthropod species with good dispersal capacities (BÁTORI & al. 2020, 2022), we might expect these factors to result in increased competition in dolines. Competition can affect the allocation of resources for producing sexual offspring (BOULAY & al. 2010), and there can be trade-offs

when competing over different resources (NEUMANN & PINTER-WOLLMAN 2022). As a result, decreased resource availability may reduce the number of gynes in a colony because they have a higher protein requirement than workers and males (BOOMSMA & ISAACS 1985, KELLER & VARGO 1993, ROSENHEIM & al. 1996). These differences were apparent between the colonies of the two microhabitats. Moreover, considering that *M. ruginodis* is adapted to colder and moister conditions (CZECHOWSKI & al. 2012), the number of suitable habitat patches for *M. ruginodis* was presumably lower on the warmer and drier plateaus, especially in drier years such as 2022. This may also contribute to the higher number of mature queens on the plateaus, because when dispersal is risky, it is more worthwhile for queens to join their natal nest (ROSENGREN & PAMILO 1983, HERBERS 1986, SOARES 2013), resulting in an elevated number of mature queens in polygynous colonies.

Contrary to our expectations, we did not find significant differences in colony size between doline and plateau microhabitats. However, the number of worker pupae was higher on the plateaus, whereas the number of worker larvae and the number of worker larvae and pupae combined was higher in dolines. This suggests that brood development is hindered by the microclimatic conditions found on the plateaus. While this may indicate the potential for larger colonies in dolines, these differences could be offset by other factors related to brood development and sociogenesis (TSCHINKEL 1993, 2011). Temperature plays an important role in the speed of insect development (DAVIDSON 1944, HONEK & KOCOUREK 1988). In August, in the dolines of the study area, the mean daily and daytime temperatures on north-facing slopes ($T_{24} = 20.1^\circ\text{C}$, $T_d = 25.7^\circ\text{C}$) were lower, whereas the mean daily and daytime relative humidities were higher on north-facing slopes ($RH_{24} = 78.4\%$, $RH_d = 68.2\%$) compared with the plateau habitats ($T_{24} = 24.0^\circ\text{C}$, $T_d = 31.8^\circ\text{C}$, $RH_{24} = 70.0\%$, $RH_d = 55.1\%$) (BÁTORI & al. 2019). Thus, in dolines the temperatures were near the optimal range for *Myrmica* ants' brood development (18 - 24°C, suppressed at temperatures above 26°C; BRIAN 1973, KIPYATKOV & LOPATINA 2002), whereas plateau temperatures were much higher. Elevated temperatures on the plateaus might hinder the egg-laying rate of queens (BRIAN & KELLY 1967), and can also lead to faster brood development and higher worker

mortality within these habitats (see TRIGOS-PERAL & al. 2024). Additionally, resource availability can influence the outcome. Dolines can provide hydrologic microrefugia for many taxa (BÁTORI & al. 2020, 2022) and support a higher ant nest density, leading to stronger competition for resources, particularly protein. Decreased resource availability may reduce the number of gynes, which have higher protein requirements than workers and males (BOOMSMA & ISAAKS 1985, KELLER & VARGO 1993, ROSENHEIM & al. 1996), as found in our study. Although the cooler and moister microclimatic conditions within dolines can favour the foraging activity of *Myrmica ruginodis*, which shifts towards lower temperatures and higher humidity in the summer period (SAVOLAINEN & VEPSÄLÄINEN 1989, LŐRINCZ & al. 2024), this seemingly cannot compensate for the lack of resources.

In dolines, the variance in worker size tended to be larger, although no difference was found in the average body size of the workers between the doline and plateau microhabitats. Higher size variance can be a colony-level advantage (HERBERS & CUNNINGHAM 1983, HÖLLDOBLER & WILSON 1990), which may be particularly important in environments where obtaining adequate amounts of nutrients is challenging due to the high levels of intra- and / or interspecific competition. Workers of different sizes can exploit various aspects of a given habitat, leading to complementary niche exploitation, thereby reducing competition and facilitating the coexistence of different species (SCHÖNING & al. 2005). Smaller workers can move more effortlessly in restricted environments (KASPARI & WEISER 1999, NOOTEN & al. 2019), such as microhabitats with taller vegetation found on the north-facing slopes and bottoms of dolines (BÁTORI & al. 2019). Conversely, larger workers can be also more efficient in catching and transporting larger prey (RETANA & CERDÁ 1994, BATCHELOR & al. 2012) and may participate in other tasks like removing obstacles while foraging (BERNADOU & al. 2016). For example, in *Lasius niger*, foragers are usually smaller than intranidal workers (OKRUTNIAK & al. 2020), whereas in wood ants, workers that gather protein food are larger than the workers collecting honeydew (ROSENGREN & SUNDSTRÖM 1987). Additionally, large wood ant workers play an important role in nest defense as they are better fighters than smaller ones (BATCHELOR & al. 2012). These findings suggest that colonies with varied worker sizes can leverage the advantages of both small and large workers, which can be very important in environments with high nest density and structural complexity, such as microhabitats in doline microrefugia. However, the interpretation of our results regarding worker size variability should be approached with caution due to the low sample size of workers sampled from single colonies.

We found that *Myrmica ruginodis* workers in dolines exhibited more pronounced aggressiveness compared with those in plateaus. While there was virtually no difference in within-individual behavioural variation across the two habitats, ants inhabiting dolines showed significantly higher among-individual variation. Our repeatability

estimates can be considered as high (in dolines) vs. low (in plateaus), based on the classic meta-analysis by BELL & al. (2009), reporting the average repeatability of flexible behavioural traits to be 0.37. Biologically, this indicates that the doline populations are composed of individuals with more distinct behaviour compared with plateau populations. One plausible reason behind this is that the restricted space and lack of resources favour individuals that are more aggressive and better competitors (SORVARI & HAKKARAINEN 2004). Such competitive environments can significantly increase the individual repeatability and interindividual variability of worker aggressiveness, showing that individual strategies can be more efficiently applied, and the variance can play an important role in increasing colony fitness (MODLMEIER & FOITZIK 2011, MAÁK & al. 2021). This is also supported by the higher worker size variability found in dolines. Moreover, such individual differences in functional and behavioural traits can also favour the division of labour (WILLS & al. 2018, TRIGOS-PERAL & al. 2023). Thus, we can assume that the consistency of aggressiveness and the enforcement of unique strategies can be decisive in dolines with buffered environments, more variable microhabitats, and higher intra- and interspecific density (BÁTORI & al. 2022).

Our study represents an important step towards a more detailed understanding of the ecological function of microrefugia and highlights the outstanding role of functional trait plasticity within an arthropod species. Our results suggest that microrefugia not only enable the survival of species with different functional traits compared with those from the surrounding matrix, but can also filter for the functional traits of a given species. Although *Myrmica ruginodis* is a cold and moisture-preferring species, it can also occur in areas where the conditions are not so suitable (CZECHOWSKI & al. 2012). However, due to the increasingly warming environmental conditions, the number of habitats within the tolerance limit of this species may decrease. Thus, the ability to survive in habitats where the abiotic conditions are suitable but facing numerous limiting biotic factors is extremely important. Survival in such habitats is made possible by the plasticity of functional traits, which can result in similar overall outcomes among populations. Because of their prominent role in many ecosystem processes, the loss of ant species can have a strong impact on the natural environment (CRIST 2009, SANDERS & VEEN 2011). Consequently, it is extremely important to identify, understand, and preserve microhabitats that may provide suitable conditions for many species for a longer period, mitigating the negative effects of environmental changes by ensuring the appropriate functioning of ecological processes, and thereby, conserving species diversity.

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Declaration on use of generative artificial intelligence tools

The authors declare that they did not utilize generative artificial intelligence tools in any part of the composition of this manuscript.

Authorship contribution statement

Bonita Ratkai: Conceptualization, Methodology, Investigation, Data curation, Formal analyses, Original draft preparation, Reviewing, and Editing; Kata Anna Bán: Investigation, Reviewing, and Editing; Kata Frei: Investigation, Reviewing, and Editing; Gergely Horváth: Formal analyses, Reviewing, and Editing; Gábor Li: Investigation, Reviewing, and Editing; Ádám Lőrincz: Investigation, Reviewing, and Editing; Gábor Lőrinczi: Investigation, Reviewing, and Editing; Fanni Pécsy: Investigation, Reviewing, and Editing; Zoltán Bátor: Conceptualization, Methodology, Investigation, Original draft preparation, Reviewing, and Editing; István Elek Maák: Conceptualization, Methodology, Investigation, Formal analyses, Original draft preparation, Reviewing, and Editing.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Data availability

Analyses reported in this article can be reproduced using the data provided at <https://zenodo.org/records/10777586>.

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