



Queen and worker phenotypic traits are associated with colony composition and environment in *Temnothorax rugatulus* (Hymenoptera: Formicidae), an ant with alternative reproductive strategies

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Abstract

Alternative reproductive strategies are often associated with distinct morphological phenotypes. Some ant species display two queen morphs: larger queens (macrogyenes) conduct mating flights followed by independent colony foundation, whereas smaller queens (microgyenes) are readopted by their mother colony. In some cases, microgyenes can evolve into social parasites that seek adoption into non-natal colonies. Here, we used morphometric measurements, behavioral experiments, chemistry, and demographic analyses to characterize queen alternative reproductive strategies in the ant *Temnothorax rugatulus* (EMERY, 1895) and question whether there is evidence for the evolution of social parasitism in microgyenes. We show that body size is differently affected by colony composition in the two queen morphs. Interestingly, worker body size is also influenced by queen morph and colony composition, and the smallest workers are found in colonies with a single microgyne. Colony composition changes across collection sites, and colonies with microgyenes are more frequent at higher elevations, suggesting that alternative reproductive strategies might be primarily associated with environmental conditions in this species. Behavioral experiments revealed a similar, low likelihood of both morphs to be accepted by non-natal colonies, which is consistent with microgyenes being a non-parasitic, reproductive morph. This finding is corroborated by similar chemical profiles between queen morphs, which are again rather influenced by colony composition. Our study highlights the association between colony composition, environmental factors, and queen dimorphism, giving more insights into the evolution of alternative reproductive strategies in ants.

Key words: Alternative reproductive strategies, queen dimorphism, morphometry, behavior, cuticular hydrocarbons, social insects.

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Introduction

Social insects are great models for the study of intraspecific polymorphism. Societies of ants, bees, and termites exhibit a reproductive division of labor, where the queens represent the main reproductive caste, while workers perform all other necessary tasks for the colony such as foraging, guarding, and brood and nestmate care (HÖLDOBLER & WILSON 1990). Reproductive and non-reproductive individuals display strong phenotypic differences, the queens being typically winged and larger than the workers. In most social insects, caste is not genetically determined but arises from phenotypic plasticity (CORONA & al. 2016).

Indeed, environmental influences during development such as different temperatures, food quality or quantity affect caste determination (LIBBRECHT & al. 2013, BERENS & al. 2015). Interestingly, beyond the typical differences between queens and workers, polymorphisms can also occur within the queen or worker caste, shedding light on the evolution and maintenance of alternative phenotypes (RIBEIRO & al. 2006).

In ants, polymorphism within the queen caste is often associated with alternative reproductive strategies (RÜPPELL & HEINZE 1999, KELLER & HEINZE 2000, WOLF

& SEPPÄ 2016). Queens differ in traits associated with reproduction and dispersal like fat content, body size, or wing presence (HAKALA & al. 2019). In some ant species, queen size is bimodally distributed. The larger queens (macrogyne) are provided with sufficient body reserves to found their colony independently, whereas the smaller queens (microgyne) seek readoption into their mother colony, and lay eggs alongside the other queens (MCINNES & TSCHINKEL 1995, RÜPPELL & al. 1998). In the latter case, workers assist queens during the dependent colony foundation, and the colony reproduces by budding or fission (PEETERS & MOLET 2009). In other ant species, primarily wingless queens evolved to not disperse but mate within or near their mother colony (FOITZIK & al. 2010, PEETERS 2012). These strategies of dependent colony foundation have evolved repeatedly in ants (CRONIN & al. 2013). They maximize queen survival by avoiding the risky solitary founding phase (MOLET & al. 2009), which can be advantageous in habitats where resources or nest sites are limited (HEINZE 1993, BOURKE & HEINZE 1994). Besides, microgyne can evolve a parasitic strategy, by seeking adoption into non-natal colonies headed by macrogyne and exploiting their workforce (FOITZIK & HEINZE 1998, KELLER & HEINZE 2000). In some cases, speciation facilitates further adaptations including the loss of the worker caste and the evolution of true inquiline, a form of obligate social parasitism (HEINZE & BUSCHINGER 1989, SAVOLAINEN & VEPSÄLÄINEN 2003, LEPPÄNEN & al. 2015, DE LA MORA & al. 2020).

In our model species *Temnothorax rugatulus* (EMERY, 1895), two queen morphs are associated with alternative reproductive strategies (RÜPPELL & al. 2001a, b, RÜPPELL & al. 2002). The large macrogyne predominantly found their colony independently, benefiting from higher fat content, and mostly residing as the sole queens in monogynous colonies. The small, worker-sized microgyne are instead seeking readoption into their mother colonies, and are thus commonly found in polygynous colonies with up to several dozens of queens. Most colonies contain queens of a single morph, but mixed colonies with both macrogyne and microgyne occur and make up about 8% of the colonies in the wild (see methods section). Microgyne exhibit a similar egg-laying rate as macrogyne in small colonies, which is most likely mediated by their higher metabolic rate, which they maintain by being fed more often by the workers (Matteo Negroni, pers. comm.). In mixed colonies, microgyne produce proportionally fewer workers and more sexual offspring than macrogyne (RÜPPELL & al. 2002), a trait which has been associated with social parasitism before (HORA & al. 2005, SCHÄR & NASH 2014).

In this study, we enrich previous work on queen alternative reproductive strategies in *Temnothorax rugatulus* (see RÜPPELL & al. 1998, RÜPPELL & al. 2001a, b, RÜPPELL & al. 2002, HEINZE & RÜPPELL 2014) by adding novel information on queen, worker, and colony traits. Using morphometric measurements, we analyzed how queen and worker body size varies with colony composition. We

then investigated an association between queen alternative reproductive strategies and environmental conditions. We predicted that microgynous colonies would be prevalent at higher elevations since microgyne use dependent colony foundation. Besides, we used readoption experiments to investigate whether microgyne are more likely to successfully intrude non-natal colonies. Such a tendency would be indicative of early steps towards the evolution of a parasitic strategy, despite the lack of genetic differentiation between the two queen morphs (RÜPPELL & al. 2001a). Finally, we extracted and analyzed the cuticular hydrocarbons from queens of the two morphs, which have been shown to differ between social morphs in *Solenopsis invicta* (KELLER & ROSS 1998), workers from different social origins in *Formica selysi* (MEUNIER & al. 2011), or social parasites (NEHRING & al. 2015, KLEEBERG & al. 2017).

Material and methods

Ant collection and maintenance: *Temnothorax rugatulus* is a small ant species distributed throughout the western part of North America. These ants inhabit high elevation coniferous forests, residing mostly in rock crevices or under stones. In August 2018, 857 colonies were collected from nine different locations in the Chiricahua Mountains (Arizona, USA, Tab. S1a, Appendix, as digital supplementary material to this article, at the journal's web pages). The ant species was identified by Susanne Foitzik in the field and later confirmed in the laboratory using a determination key (MACKAY 2000). In the laboratory, each colony was kept in a box (9.7 × 9.7 × 2.9 cm) with three chambers connected by holes. Each colony was provided with a microscopic slide nest covered with a red foil to block the light and a lid. Ant colonies were maintained at 21 °C and 70% humidity with a 12:12 light:dark cycle and fed weekly with half a cricket and honey. To increase ants' activity for the behavioral experiments (see below), the ants were moved to 25 °C and 70% humidity with a 12:12 light:dark cycle. These colonies were fed weekly during the experimental period with honey and an artificial diet composed of honey, eggs, agar, and crickets.

Morphometric measurements: After transfer to the laboratory, the number of workers in each colony was counted, and the head width, thorax width, and thorax length of all queens from all colonies (N = 2227 individuals) were measured. Queens were assigned to the macrogyne or microgyne morph based on their thorax width and additional criteria (see detailed protocol in digital supplementary material). Additionally, the head and thorax width of two nurses (i.e., workers close to the brood) and two foragers (i.e., workers collecting food) from twelve colonies of each colony morph (macrogynous, microgynous) and social structure (monogynous, pure polygynous) were measured, using a full factorial design (N = 192 individuals). Head width is the most used proxy for body size in workers. Measuring the thorax width was also interesting since macrogyne and microgyne strongly differ in this trait. Photos were taken of live ants, immobilized in modeling clay (Play-Doh) on a wooden ball

under a Leica stereomicroscope (magnification $\times 20$), and measures were done using the Leica software LAS version 4.5. The following size index by RÜPPELL & al. (1998) was used as a proxy for queen body size:

$$\text{size index (mm)} = \frac{\sqrt{\text{thorax length} * \text{thorax width} + \text{head width}}}{2}$$

In their natural environment, queens of the two morphs occur by themselves in monogynous colonies or in polygynous colonies with other queens of their own morph. Occasionally, they also occur in large, mixed colonies with queens of both morphs. Thus, the influence of queen morph (macrogyne, microgyne) in interaction with colony composition (monogynous, pure polygynous, mixed polygynous) on queen body size was investigated using a linear mixed-effects model with the R package “lme4” (BATES & al. 2015). Post-hoc Tukey comparisons were then carried out using the package “multcomp” (HOTHORN & al. 2008). Linear mixed-effects models were also used to investigate the influence of colony morph (macrogynous, microgynous), social structure (monogynous, pure polygynous), and their interaction on worker head and thorax widths. Colony identification (ID) was used as a random factor in all models to account for inter-colony variability. The models’ fit was assessed using visual inspections of the residual distributions. Alpha was set at 0.05 for all statistical tests. All analyses were conducted in R version 3.5.1 (R CORE TEAM 2018).

Demographic analyses: To address whether the ecological background influences *Temnothorax rugatulus* colonies, the data were combined with additional data from a previous collection trip in August 2015, when 557 ant colonies were collected at 15 sites throughout the Chiricahua Mountains (Arizona, USA, Tab. S1b). The number of colonies of each composition is summarized in Table S2. First, the collection site influence on colony composition (monogynous macrogynous, polygynous macrogynous, monogynous microgynous, polygynous microgynous, and polygynous mixed) was investigated using a Chi-square test. Then, the effect of the collection sites’ elevation on the proportion of colonies containing microgynes, as well as the proportion of polygynous colonies, was tested using linear models. Collection sites with fewer than eight colonies were removed from the models to avoid unreliable proportions. Differences in the number of workers per queen depending on the colony morph (macrogynous, microgynous) were also investigated using a linear mixed-effects model with the collection site as a random factor. Here, the terms macrogynous and microgynous colonies encompass both monogynous and polygynous colonies. The models’ fit was assessed using visual inspections of the residual distributions.

Behavioral experiments: Readoption experiments were carried out to test the microgynes’ ability to successfully intrude non-natal colonies, in comparison with macrogynes (Fig. S1). Initially, 32 source colonies containing queens of both morphs were used. From each source colony, a macrogyne and a microgyne were tested.

The tested queens were marked with wire loops (0.02 mm Elektrisola, Eckenhausen, Germany) between the petiole and post-petiole. Each queen was tested for each of four host colony compositions, one after the other in a pseudo-randomized order. The queen was confronted with (i) her natal colony, (ii) a non-natal monogynous colony with a single macrogyne, (iii) a non-natal polygynous colony with multiple macrogynes, and (iv) a non-natal polygynous colony with multiple microgynes. In total, 20 host colonies of each colony composition were used. Host colonies with a single microgyne could not be used because of their limited number.

The host colony was placed in the left chamber of a three-chambered box. The left chamber (2.9 \times 9.7 cm) served as an arena for the experiment and was isolated from the rest of the box using tape to cover the connecting hole. The tested queen was placed about 1 cm in front of the nest entrance of the host colony using clean forceps. Scans were performed every five minutes during the first hour following the start of the trial (N = 12 scans per trial). For each scan during the first hour, the location of the queen (inside the nest, outside the nest) and the type of interaction between queen and workers (aggression, grooming) were reported. After 24 hours, the location of the queen and whether she was alive or not was reported, before returning the queen to her colony. Each queen had a 5-day break in between each trial. It was not possible to collect the data blind since queen morph can be identified visually. Three source colonies were removed from the experiment because the queen died before being tested or lost her wire (final N = 29). Because the queens were often mutilated by the workers, the death rate was higher than expected, and the final number of trials was N = 64 for the macrogynes and N = 42 for the microgynes (Tab. S3).

First, the likelihood of queens of both morphs to be accepted by their natal colony compared with a non-natal one was investigated. Generalized linear mixed-effects models (GLMMs, binomial family) were used to test for the effect of colony origin (natal, non-natal) on (i) the queen location after one hour and the proportion of scans where at least one (ii) aggression or (iii) grooming event occurred during the first hour. Queen ID was always used as a random factor because each source colony provided one queen of each morph. The GLMMs were tested for overdispersion using the package “DHARMA” (HARTIG 2020). The survival and location of the queen 24 hours after being introduced to the host colony was tested using a Fisher test, because the survival and success rates of queens entering their natal colony were 100% for this analysis and thus, binomial models did not converge. Dead queens were removed from the data set when analyzing the effect of colony origin on the location after 24 hours.

Similar binomial models with queen ID as a random factor and model inspection methods were used to investigate whether the two queen morphs had different likelihoods of successfully intruding non-natal colonies, and whether the composition of these non-natal colonies played a role. The effects of queen morph (macrogyne, mi-

crogyne), colony composition (monogynous macrogynous, polygynous macrogynous, and polygynous microgynous), and their interaction were tested on (i) the queen location after one hour and the proportion of scans where at least one (ii) aggression or (iii) grooming event occurred during the first hour and queen (iv) survival and (v) location 24 hours after the introduction to the host colony. Only non-natal colonies were kept as host colonies here. Using colony composition as a single response variable was the only way to analyze the data rigorously since the experimental design was not fully factorial, due to the lack of monogynous microgynous host colonies. Additionally, none of the queens could be used for all the four trials as originally planned due to high mutilation and death rates. Consequently, host colonies were used variably, between one and four times. Thus, independent data sets were created to test for the effects of colony origin and queen morph in interaction with colony composition, where only the host colonies used once were kept and one trial for the host colonies used multiple times was randomly selected. The order of trial was included as a covariate in all the models to test for a potential effect on the queens' performance, and then removed since no significant effect was found.

Chemical analyses: Cuticular hydrocarbon (CHC) profiles of macrogynes and microgynes were analyzed using two separate data sets: (A) CHC profiles of ten macrogynes and ten microgynes from the same mixed colonies, and macrogynes from ten pure macrogynous colonies (data collected in September and October 2019) and (B) CHC profiles of ten macrogynes and ten microgynes from pure colonies (data collected in February 2020) (Tab. S4). Due to potential experimenter and biological biases, the analysis of these data sets was done separately.

For both data sets, gas chromatography-mass spectrometry (GC-MS) was used to determine whether the cuticular hydrocarbon profiles of macrogynes and microgynes differed. After spending two weeks under the same conditions (25 °C and 70% humidity with a 12:12 light:dark cycle and fed weekly with honey and half a cricket), the queens were individually frozen in glass vials and stored at -20 °C. To extract the CHCs, each ant was covered in n-hexane for ten minutes and a standard (100 ng n-octadecane solved in 10 µl n-heptane) was added for absolute quantification of the CHCs. The extracts were transferred to micro inserts, evaporated under a nitrogen stream, and injected into a gas chromatograph (7890A, Agilent Technologies, Santa Clara, CA, USA) coupled to a mass selective detector (5975C, Agilent). A Zebron Inferno DB5-MS capillary column (length 30 m, diameter 0.25 mm, 0.25 µm coating, Phenomenex Ltd, Aschaffenburg, Germany) was used as stationary phase and helium with a flow rate of 1.2 ml / min as a carrier gas. The temperature was initially at 60 °C, then increased to 200 °C at 60 °C / min and finally to 320 °C at 4 °C / min, where it was kept constant for ten minutes. The mass spectrometer had an ionization voltage of 70 eV and fragments were scanned 40 to 550 m / z. Manual integration of the derived peaks was conducted using the software MSD ChemStation E02.02 (AGILENT TECHNOLOGIES

2008). The integrated peaks were then manually aligned, and hydrocarbons were identified using their retention index as well as diagnostic ions in Microsoft Excel.

One outlier was removed in each data set because their CHC composition was strongly deviating from all the other samples in multivariate analysis plots, and was thus considered an artifact. Differences between CHC profiles of the queens were tested with a permutational MANOVA (999 iterations) using the programs PRIMER 6 (version 6.1.14; CLARKE & GORLEY 2006) and PERMANOVA+ (version 1.0.4; ANDERSON & al. 2008) (both Primer-E Ltd., Plymouth, UK). Queen morph was used as a fixed factor in both the analyses of the data set A (macrogyne, microgyne) and the data set B (macrogyne pure colony, macrogyne mixed colony, and microgyne pure colony). For the analysis of the data set A, colony ID was used as a random factor and pairwise tests were carried out. Using the data set A, differences in the proportion of alkanes between the two queen morphs were additionally investigated using a linear mixed-effects model with colony ID as a random factor. The model fit was assessed using visual inspections of the residual distributions.

Results

Morphometry: We calculated a size index according to RÜPPELL & al. (1998) as a proxy for queen body size and obtained a clear bimodal distribution (N = 1784 macrogynes, 0.819 mm ± 0.034 mm; N = 387 microgynes, 0.697 mm ± 0.031 mm; Fig. 1). Body size changed depending on queen morph ($X^2 = 2563.029$, degrees of freedom (df) = 1, $p < 0.001$) and colony composition ($X^2 = 130.602$, df = 2, $p < 0.001$), and we found a significant effect of the interaction between the factors ($X^2 = 58.656$, df = 2, $p < 0.001$; Fig. 2, Tab. S5). Macrogynes from multiple-queen colonies showed a reduction in body size compared with macrogynes from single-queen colonies ($z = -9.387$, $p < 0.001$) and were even smaller when sharing their colony with microgynes ($z = -9.401$, $p < 0.001$). Interestingly, a different pattern was observed in microgynes, which were significantly smaller in monogynous ($z = 2.770$, $p < 0.05$) and pure polygynous colonies that contained only micro-

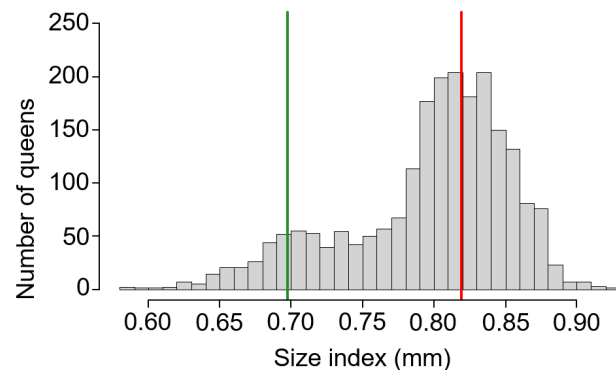


Fig. 1: Queen body size distribution in *Temnothorax rugatulus* showing the mean body size of macrogynes (red) and microgynes (green).

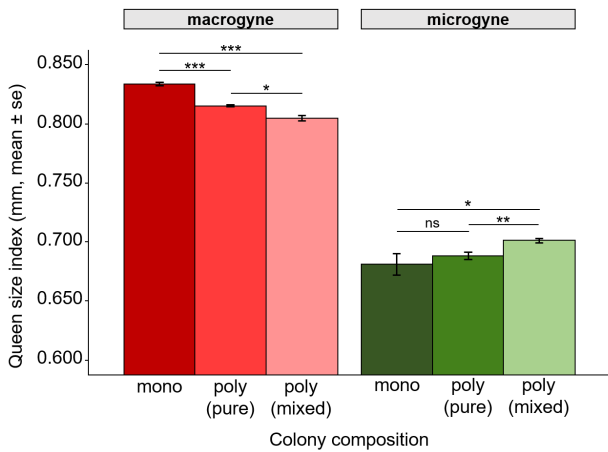


Fig. 2: Interactive effects of queen morph and colony composition (mono = monogynous and poly = polygynous) on queen body size. The levels of significance are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. To improve readability, only the significance of intra-morph comparisons is provided.

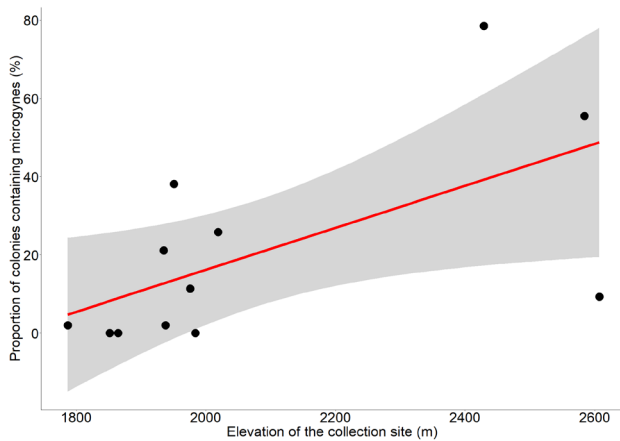


Fig. 4: Proportion of colonies containing microgynes depending on the elevation of the collection site. Each dot represents one collection site.

gynes ($z = -3.340$, $p < 0.01$), compared with mixed colonies with both morphs.

Worker head and thorax widths were highly correlated ($F = 289.7$, $df = 1$, $r^2 = 0.602$, $p < 0.001$; Fig. S2). We found a significant effect of social structure on worker head width ($X^2 = 8.020$, $df = 1$, $p < 0.01$), and workers from polygynous colonies had larger heads compared with workers from monogynous colonies. Workers from polygynous colonies also tended to have larger thoraces ($X^2 = 3.699$, $df = 1$, $p = 0.054$). We then compared thorax width of workers from macrogynous and microgynous colonies and found a strong effect of colony morph on thorax width ($X^2 = 20.318$, $df = 1$, $p < 0.001$), and workers from microgynous colonies, that is, produced by microgynes, had smaller thoraces than those from macrogynous colonies. We also detected a significant interaction between social structure and colony morph on thorax width ($X^2 = 5.671$, $df = 1$, $p < 0.05$; Fig. 3, Tab. S6). Workers produced by microgynes had

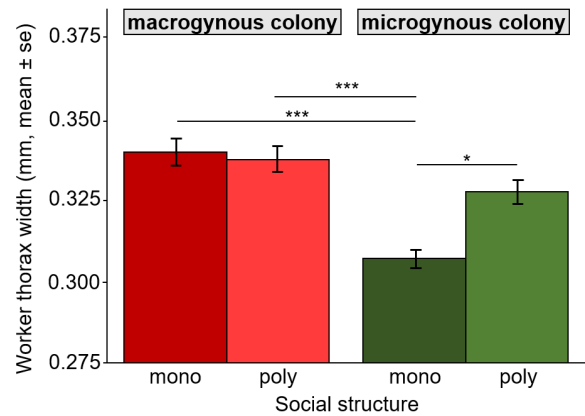


Fig. 3: Interactive effects of colony morph and social structure (mono = monogynous and poly = polygynous) on worker body size. The levels of significance are indicated as follows: * $p < 0.05$, *** $p < 0.001$.

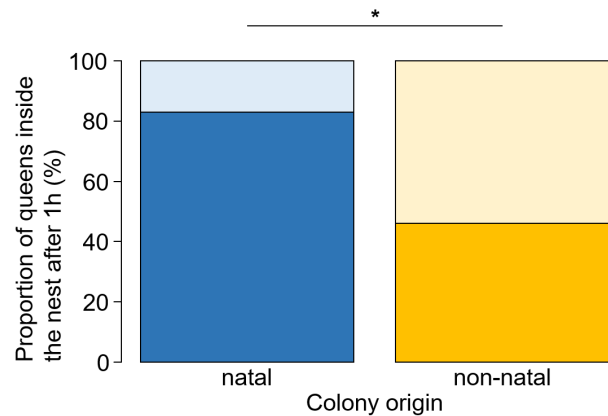


Fig. 5: Proportion of queens found inside the nest (darker shade) of natal and non-natal colonies one hour after their introduction to the colony. The level of significance is indicated as follows: * $p < 0.05$.

larger thoraces in polygynous compared with monogynous colonies ($z = 3.044$, $p < 0.05$), while social structure did not influence worker thorax width in macrogynous colonies.

Demography: The Chiricahua Mountains in South-eastern Arizona rise to 2976 meters and *Temnothorax rugatulus* colonies reside in rock crevices on the harsh mountaintops as well as in the valleys. First, we tested whether colony composition changes with the site of collection and did find differences between sites (Pearson's Chi-squared test, $X^2 = 366.09$, $df = 56$, $p < 0.001$; Fig. S3). Then, we tested whether the elevation of the collection sites had an influence on queen morph and colony composition in *T. rugatulus* colonies. Indeed, the proportion of colonies containing microgynes increased with elevation ($F = 6.116$, $df = 1$, $p < 0.05$; Fig. 4). However, we did not find an effect of elevation on the proportion of monogynous versus polygynous colonies ($F = 3.249$, $df = 1$, $p = 0.102$). Finally, queens from microgynous colonies had fewer workers compared with queens from macrogynous colonies ($X^2 = 33.82$, $df = 1$, $p < 0.001$).

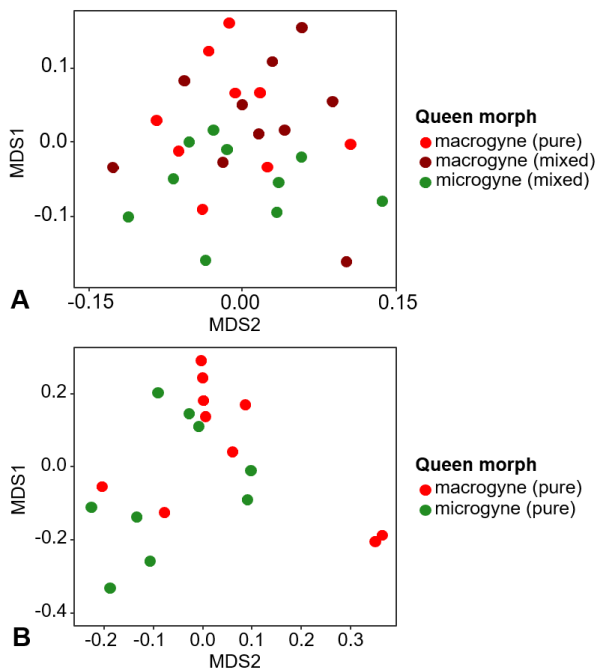


Fig. 6: Non-Metric Multidimensional Scaling (NMDS) plots showing the cuticular hydrocarbon (CHC) profiles of (A) macrogyne from pure and mixed colonies and microgyne from mixed colonies and (B) macrogyne and microgyne from pure colonies. Each dot represents one individual.

Behavior: First, we tested whether queens, regardless of their morph, were more likely to be accepted by their natal colony than by a non-natal colony. After one hour, queens were more often found inside the nest of their natal colony compared with non-natal colonies ($X^2 = 4.712$, $df = 1$, $p < 0.05$; Fig. 5). They were also less often attacked ($X^2 = 6.938$, $d = 1$, $p < 0.01$) and more often groomed ($X^2 = 7.231$, $df = 1$, $p < 0.01$) by their own workers. After 24 hours, all queens that entered their natal colony were found alive inside their colony, whereas 37% of the queens which tried to enter a non-natal colony were found dead (Fisher's Exact Test, $p < 0.05$). Among the living queens, only 55% were found inside the nest of the non-natal colony, and the remaining 45% were found outside the nest (Fisher's Exact Test, $p < 0.01$).

We then tested whether queen morph influenced the success of intruding non-natal colonies and whether the colony composition (monogynous macrogynous, polygynous macrogynous or microgynous) of the host colonies played a role. Queen morph did not affect the likelihood of being found inside a non-natal colony after one hour ($X^2 = 0.002$, $df = 1$, $p = 0.969$), nor did the composition of the non-natal colony ($X^2 = 1.641$, $df = 2$, $p = 0.440$). We did not find an interaction between queen morph and colony composition on the success rate of the queens ($X^2 = 2.052$, $df = 2$, $p = 0.359$). Similar patterns were found regarding the aggression and grooming behaviors of the workers towards the intruding queens since queen morph, colony composition, and their interaction did not have significant effects (Tab. S7). The results were similar after 24 hours

(Tabs. S7 and S8). In summary, microgyne and macrogyne were both more likely to be accepted by their natal colony and had a similar, low likelihood of successfully intruding a non-natal colony.

Chemistry: We investigated potential differences in the cuticular hydrocarbon (CHC) profiles of macrogyne and microgyne (Tab. S9 and Fig. S4) from different colony compositions. Cuticular hydrocarbon profiles of microgyne and macrogyne from the same mixed colonies did not differ (PERMANOVA: $t = 0.762$, $df = 18$, $p = 0.770$; Fig. 6A), nor was there a difference in CHC profiles of microgyne and macrogyne from different pure colonies (PERMANOVA: $t = 1.898$, $df = 1$, $p = 0.135$; Fig. 6B). However, macrogyne from pure colonies were different from microgyne of mixed colonies ($t = 1.897$, $df = 17$, $p < 0.01$; Fig. 6A) and also tended to differ from macrogyne of mixed colonies ($t = 1.522$, $df = 17$, $p = 0.051$; Fig. 6A). Macrogyne and microgyne did not differ in their proportion of alkanes ($X^2 = 0.001$, $df = 1$, $p = 0.970$).

Discussion

Alternative reproductive strategies evolve when environmental or social conditions fluctuate, and are often associated with variation in phenotypic traits matching the respective strategy (GROSS 1996, TABORSKY & BROCKMANN 2010). Here, we used multiple approaches to characterize queen alternative reproductive strategies in *Temnothorax rugatulus*. Our results shed light on different traits associated with queen alternative reproductive strategies, which are extending to the worker caste and could potentially be associated with environmental conditions. However, we found no evidence of socially parasitic tendencies in microgyne.

In ants, dependent colony foundation usually leads to functional polygyny where several queens reproduce in a single colony, and is often associated with a reduction in queen body size since readoption and budding do not require large body reserves (KELLER 1995, LIBBRECHT & KRONAUER 2014, WOLF & SEPPÄ 2016). Our data confirm this size reduction in polygynous macrogyne. However, we observe a different pattern in microgyne, which are larger when occurring in mixed colonies with macrogyne, compared with monogynous and polygynous colonies with microgyne only. Since body size is correlated with fecundity in insects (HONEK 1993), we can hypothesize that both queen morphs display fitness optima when living in the colony composition associated with their reproductive strategy – monogynous colonies for macrogyne versus mixed polygynous colonies for microgyne – and where they are most often found in nature. Supporting this hypothesis, microgyne produce fewer and smaller workers in pure microgynous colonies, while they can keep up their egg-laying rate with macrogyne in mixed colonies (Matteo Negrone, pers. comm.). Also, monogynous microgynous colonies are relatively rare in nature (2.7% of our collected colonies).

Our worker data reflects the relationship between body size, colony composition, and fitness observed in queens since the smallest workers (i.e., workers with the smallest

thoraces) are found in colonies with a single microgyne. Interestingly, monogynous microgynes are also the smallest queens. It has been previously suggested that maternal effects are responsible for body size transmission in *Temnothorax rugatulus* (see RÜPPELL & al. 2001b), which is supported by our data showing that the smallest queens produce the smallest workers. Similar results are found in *Myrmica ruginodis*, where microgynes produce smaller workers (ELMES 1991). In our species, however, we cannot rule out that both queens and workers are smaller in monogynous microgynous colonies because of resource limitation, which could be linked to the poor colony fitness discussed above. In general, it seems that worker size is rather plastic in *T. rugatulus*, which contrasts with other species with queen polymorphism such as *Solenopsis invicta*, where alleles on the gene Gp-9 have been shown to affect the body mass of field-collected workers (GOODISMAN & al. 1999). Finally, our data revealed that workers from polygynous colonies have larger heads, which could suggest stronger territoriality in habitats where polygynous colonies are denser (ADAMS 2016), and agonistic interactions with unrelated individuals consequently more frequent.

Dependent colony foundation can be associated with ecological factors (HEINZE 1993, BOURKE & HEINZE 1994) and colonies of *Temnothorax rugatulus* inhabit diverse habitats from valleys to mountaintops. Thus, we decided to investigate whether the ecological background influences different colony traits in our model species. Interestingly, colony composition strongly varies with the site of collection. Since our collection sites appeared to be ecologically different, we investigated whether the site effect we found could be driven by differences in elevation, and found that colonies containing microgynes occur more often at higher elevations. This finding goes in a similar direction to what was previously found in this species (RÜPPELL & al. 2001a, HEINZE & RÜPPELL 2014), although we were unable to demonstrate a significant increase in the proportion of polygynous colonies with elevation, most likely because our range of elevations was much smaller. Habitats at higher elevations are most likely harsher and resources might be limited, leading to food restriction for the growing larvae, which in turn might result in smaller queens producing smaller workers (RÜPPELL & al. 2001b). A similar strategy is found in *Myrmica ruginodis*, where the polygynous microgyne form is more common in rapidly changing habitats (SEPPÄ & al. 1995), highlighting once more the similarity with our system in terms of reproductive strategies. Thus, producing smaller queens and workers might be an advantageous strategy for colonies inhabiting difficult environments. However, we should keep in mind that those results might be specific to the population we studied (i.e., Chiricahua mountains) since microgynes are less common in other populations of *T. rugatulus* (Susanne Foitzik, pers. comm.), and other environmental factors correlating with elevation might be involved in shaping colony traits (FOITZIK & al. 2004).

Along with previous studies, we provide insights into queen size dimorphism in association with reproductive

strategies in *Temnothorax rugatulus* (see RÜPPELL & al. 1998, RÜPPELL & al. 2001a, b, RÜPPELL & al. 2002, HEINZE & RÜPPELL 2014), and our data suggest that the production of microgynes could be an adaptation to difficult environmental conditions. However, the existence of a microgyne morph has been previously discussed as a route to social parasitism in social insects (WOLF & SEPPÄ 2016) and parasitic microgynes are found in several ant species like *Ectatomma tuberculatum* (see HORA & al. 2005) or *Myrmica rubra* (SCHÄR & NASH 2014). Moreover, in some ant species, the role of microgynes is not clear yet (LENOIR & al. 2010). For these reasons, we investigated queens' behavior in readoption experiments and analyzed their chemistry, to better characterize the two queen morphs and rule out the potential parasitic nature of microgynes.

Our behavioral experiments revealed that both queen morphs were more likely to be accepted by their natal colony compared with a non-natal colony. Not surprisingly, *Temnothorax rugatulus* ants seem to possess an effective nestmate recognition system and do not accept unrelated individuals in their colony, like many other ant species (STURGIS & GORDON 2012). Microgynes did not more likely successfully intrude non-natal colonies compared with macrogynes, probably because they are usually readopted by their mother colony (RÜPPELL & al. 2001a, 2002). Whereas in the ant *Myrmica rubra*, which displays intraspecific social parasitism, the survival rate of microgynes intruding non-natal colonies is high (SCHÄR & NASH 2014). Our cuticular hydrocarbon (CHC) analyses also give evidence for microgynes being a reproductive morph rather than a socially parasitic one since they do not exhibit a higher proportion of alkanes, like some parasitic ants do to avoid recognition by their hosts (KELLER & ROSS 1998, NEHRING & al. 2015).

Additionally, we did not find evidence that CHC profiles of microgynes differ from macrogynes, as opposed to *Solenopsis invicta* and *Formica selysi* where social morphs (*S. invicta*) and workers from different social origins (*F. selysi*) have a different chemistry (KELLER & ROSS 1998, MEUNIER & al. 2011). In those species, queen morph and colony composition are determined by a so-called social chromosome (WANG & al. 2013, PURCELL & al. 2014, BRELSFORD & al. 2020, YAN & al. 2020). The difference in our results could be explained by the genetic proximity of the two queen morphs in *Temnothorax rugatulus* (see RÜPPELL & al. 2001a). Interestingly, CHC profiles seemed rather to be influenced by colony composition (pure colonies with only one queen morph versus mixed colonies where the two queen morphs co-occur). Whether mixed colonies have different chemical signatures compared with pure colonies due to the mix of queens from different morphs and workers from different queen morphs, or whether these differences are due to environmental effects (i.e., mixed colonies are more frequent at higher elevations) still needs to be elucidated. Currently, little is known about the respective contribution of genetics and environmental factors to CHC profiles (MENZEL & al. 2017a), but environmental conditions, especially humidity

and temperature, affect CHC profiles and their plasticity (MENZEL & al. 2017b, SPRENGER & MENZEL 2020).

Using multiple approaches, we shed light on a combination of traits characterizing queen alternative reproductive strategies in the ant *Temnothorax rugatulus* and confirm earlier work on the role of microgynes in that species. These traits seem tightly linked to colony composition, and we propose that both queen morphs show fitness optima in their respective most frequent colony composition: monogynous colonies for macrogynes and mixed polygynous colonies for microgynes. Interestingly, colony composition not only affects queen traits but also extends to the worker caste. As we found no evidence for parasitic strategies of microgynes and revealed a link between queen alternative reproductive strategies and environmental conditions, we suggest that colonies with multiple queens including microgynes have evolved as an adaptation to harsher environments (i.e., higher elevations). Ultimately, macrogynes and microgynes in *T. rugatulus* could potentially evolve towards becoming different ecotypes if the gene flow between the two morphs starts to diminish (NOSIL 2012, WOLF & SEPPÄ 2016). To summarize our findings, we highlighted the important role of colony composition and environmental factors in association with phenotypical traits linked to queen alternative reproductive strategies in *T. rugatulus*, pointing to the importance of ecology for the evolution of reproductive strategies in social insects.

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