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COLONY SIZE AND NUMBER OF WORKERS OUTSIDE THE NEST SITE OF THE ACORN ANT *TEMNOTHORAX CRASSISPINUS* UNDER ARTIFICIAL CONDITIONS

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ABSTRACT: Information on the location of ant nests and the possibility of estimating the colony size without opening the natural nest sites would be useful during many studies. This paper describes a trial for estimating the colony size of acorn ants of the genus *Temnothorax* without opening and destroying the potential nest sites. During the study, in September and October, acorns were collected and transferred to a laboratory. Next, after spending at least 24 hours in the refrigerator, each acorn was situated in a separate square Petri dish with a drop of honey and observed for 90 minutes. During this period, workers outside acorns were counted. Then, the acorns were opened, and ants counted. The method turned out well for distinguishing which collected acorns contain ant colonies; however, it was not possible to estimate the real size of the colony without opening the acorns. The method could be useful for a study in which information about the presence of an ant colony in certain potential nest sites is important.

KEY WORDS: Formicidae, technique, ants, cavity-nesting ant.

Introduction

Studies on ants frequently demand a transfer of the ant colonies to artificial nest sites (Czechowski & Pisarski 1992). However, the parameters of artificial and natural nesting sizes could differ; for example, the physical parameters, e.g., humidity, as well as the presence of microorganisms are different in artificial nest sites (cf. Mitrus 2021), and these parameters could affect the mortality of workers and the colony development. Therefore, the possibility of using natural nest sites and applying a method for estimating the colony size without opening the potential nest sites would be useful for numerous studies.

Ants of the genus *Temnothorax* are used in many studies on social insect ecology, behaviour, and the evolution of life histories (Heinze 2006, Dornhaus et al. 2009, Pratt 2010). This genus is a suitable model because of the small number of workers in a colony, the small body size, and the possibility to easily rear the colonies, e.g., via the possibility of keeping a whole colony in a Petri dish and feeding the ants with dead invertebrates and honey (Czechowski & Pisarski 1992, Heinze 2006). The ants typically do not construct nest sites, but use the available cavities. Depending on the species, they may inhabit cavities in the dry branches of living trees, rocks or fallen twigs; they can also nest under stones, in acorns and directly in the ground (Czechowski et al. 2012, Prebus 2017).

For laboratory studies using *Temnothorax* ants, when the ant colonies are collected, the natural nest sites are typically destroyed; for example, acorns potentially inhabited by the ants are opened when looking for the colonies. Next, the workers are collected, and the colonies are transferred to plastic boxes containing an artificial nest cavity – such a cavity could be prepared between two microscope slides (Czechowski & Pisarski 1992, Pratt 2005, Mitrus 2021). Many field experiments using the *Temnothorax* ants also demand the use of artificial nest sites. For such field studies, artificial nest cavities could be fashioned in wood or pieces of cork, as the *Temnothorax* ants willingly accept such sites (Foitzik et al. 2003, Honorio et al. 2020, Mitrus 2021). For example, such artificial nest sites were used during a study on the overwintering survival rate (Mitrus 2013, 2016, Honorio et al. 2021). However, such artificial sites are stronger than empty acorns or small twigs, and thus, the mortality of whole colonies caused by predation or by destroying whole nest sites could differ.

The current study aimed to develop and test a method to estimate the colony size of the acorn ants without opening and destroying the potential nest sites, i.e., old acorns collected on the ground in a forest. The hypothesis that the number of acorn ants feeding outside the nest is related to the colony size was tested.

Material and method

The species used during the study, the acorn ant *Temnothorax crassispinus* (Karawajew, 1926), mostly inhabits acorns and small fallen sticks (Czechowski et al. 2012, Seifert 2018), where the larvae of other insects have bored cavities (Foitzik et al. 2004, Myczko et al. 2017). It lives in light coniferous and mixed forests and is present throughout Central and Eastern Europe. Colonies of the species are small, typically ranging from a few dozen to about 200 workers, while the workers are approx. 2-4 mm in size (Czechowski et al. 2012, Seifert 2018).

For this study, 49 acorns of the common oak *Quercus robur* L. were collected on 6 September 2021, and 50 acorns were collected on 19 October 2021, in a mixed forest with a

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few oaks (Poland, Opole District, 50.6247N, 18.1090E; and 50.5814N, 18.1822E, respectively). In these areas, a high density of *T. crassispinus* colonies had been discovered during previous studies. Each of the collected acorns had a hole, probably caused by beetle larvae activity, or had other damage like cracks. Based on previous experience, such acorns frequently contain colonies of the ant species. 33 of the 49 acorns collected in September had a hole, and 26 of the 49 acorns had cracks (ten of them had both a hole and a crack); while 43 of the 50 acorns collected in October had a hole and nine of the 50 had cracks (two of them had both a hole and a crack). Each acorn was placed separately into a small, tightly closed plastic box, and was transported to a laboratory. Then, the boxes with the acorns were kept in a refrigerator at a temperature of ca. 7°C. The next day, after spending more than 24 hours in the refrigerator, the boxes were opened and each acorn was situated in a separate square Petri dish (10.2 cm × 10.2 cm × 1.9 cm). In the dish, about 3.5 cm from the acorn, a small metal circle with a drop of honey was situated. The dishes were kept at a temperature of ca. 22°C, and were observed for 90 minutes. The observations started immediately after acorns were situated in the Petri dishes. Every 15 minutes, i.e. six times, the workers outside acorn (i.e., the ants feeding on the honey, walking on the acorn, and just in the Petri dishes) were counted. The observations finished after 90 minutes, as after such a period, the ants had completely eaten the honey in some dishes. As a result, the highest number of simultaneously observed ant workers over 90 min census was used.

The acorns' length and average width were measured to the nearest 0.1 mm. The acorn volume was estimated from the following formula: $4/3 \times \pi \times (\text{length} / 2) \times (\text{width} / 2) \times (\text{width} / 2)$. However, it was impossible to precisely measure three of the acorns due to damage – the data on the size of such acorns was not used in further analyses. Then, the acorns were carefully opened, and the ants were captured with an aspirator and counted. In the case of an acorn containing several workers only, and where no eggs or pupae were found, they were classified as 'sites containing only a few individuals'.

Using the two-way analysis of variance, it was checked if the volume of acorns was similar in acorns contained colonies vs. acorns with no ants, and compared volume of acorns collected in September vs. October. The assumptions for normality and the homogeneity of variance were checked prior to the analysis; the distributions of data were skewed; thus, they were log-transformed before the analysis – the log10 function was used. As there were no differences in volumes of acorns with ant colonies and containing no ants ($F = 0.49$, $df = 1$, $p = 0.48$), and between acorns collected in September and October ($F = 1.61$, $df = 1$, $p = 0.21$), data gathered in both months were analysed together.

The Spearman's rank correlation was used to assess the relations between the maximum simultaneously observed ant workers over 90 min census in a Petri dish and the number of workers found in an acorn, as the data distributions were skewed. For the acorns containing the ant colonies, the Spearman's rank correlation was also used to assess the relationship between the volume of the acorn and the number of workers in the colony.

All statistical analyses were carried out using the Statistica, ver. 13 (TIBCO 2017) software package. All the probability values shown are two-tailed.

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Results

Colonies of *Temnothorax crassispinus* were found in 23 of the 99 collected acorns, with another seven acorns containing only a few individuals. No individuals of other ant species in the acorns were found. In the 49 acorns collected in September, 13 colonies were found (11-284 workers; two queenless and 11 queenright ones); in five acorns, 1-4 workers were found. In the 50 acorns collected on October, 10 colonies were found (7-257 workers; two queenless, seven with one queen, and one with two queens), while in two acorns contained a single worker each.

When the acorns were situated in the Petri dishes, ant workers were observed in 18/49 and 10/50 of the dishes, for the acorns collected in September and October, respectively. 0-18 workers were simultaneously noticed in different dishes. During the 90 min observation, no workers were observed in only two of the 30 acorns (i.e., 23 with the ant colonies, and the next seven containing only a few individuals) later determined to contain ants: for a colony containing a queen and seven workers, and for an acorn inside which one worker was later found; in both cases, these were acorns collected in October. The highest simultaneously observed number of workers in the arena of the Petri dish was typically noticed after 45 or 60 min, while the first worker was observed before 30 min, but rarely earlier than 15 min, from the beginning of the observation.

For all the acorns containing any ants, the maximum number of simultaneously observed workers was correlated with the number of ants in the collected acorn (the Spearman's rank correlation, $R_s = 0.68$, $N = 30$, $p < 0.0001$; Fig. 1). For the ant colonies, there was correlation between the volume of the acorn and the number of workers ($R_s = 0.52$, $N = 22$, $p = 0.012$).

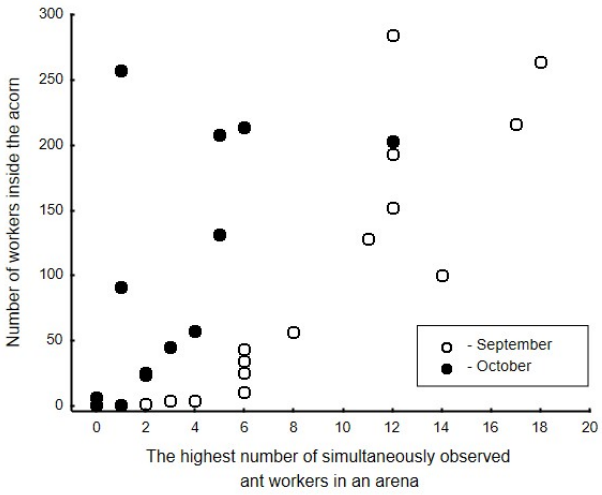

Figure 1. Relation between the highest number of simultaneously observed ant workers in an arena and number of workers inside the acorn, for the acorn ant *Temnothorax crassispinus*. An acorn with a hole or a crack was situated in a Petri dish (10.2 cm × 10.2 cm), and the workers outside the acorn were counted every 15 minutes for an observation period of 90 minutes. Additionally, a drop of honey was available for the ants in the arena. The data for September and October are marked, however, all the data analysed together (see text).

Discussion

Opening an ant nest cavity will typically destroy it, causing stress to the animals. Thus, estimating the colony size without opening the natural nest sites would be useful. The results of this study showed that the described method is a good technique for distinguishing which collected acorns contain ant colonies. However, the proposed method does not allow estimating the real size of the colony. For example, in October, in an arena, a single worker was observed for an acorn with one worker and for acorns containing queenright colonies with 91 and 257 workers, respectively.

Colonies differ in activity because of different reasons, e.g., temperature. Relocation to a laboratory could affect the ant activity, as well. Thus, keeping the colonies in the refrigerator aimed to standardize the conditions

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for the study. However, for example, colonies collected in the field might differ in food supply, and such initial differences could explain part of the observed variability. This research was performed in September and October, as in autumn, colonies are frequently collected for studies on overwintering ants (Mitrus 2013, 2016, Honorio et al. 2021). However, the season could have an influence on the results. During another study, in the early spring before the ant activity started (at the beginning of March), using – as was later determined – 14 acorns containing ant colonies, no workers were observed in the arena of the Petri dishes during the 90 min observation (unpublished results). Thus, the period of 90 min was probably too short to start the ant workers' activity. As these acorns were collected before the ant activity started, it is possible that some of the holes used by the ants as entrances to the cavities were blocked, meaning that the ants would need more time to emerge from the nest sites.

During previous studies, it was found that the nest site size was correlated with the colony size for *T. nylanderi* (Forster, 1850) (Foitzik & Heinze 1998); however, the volume of the nest site, not just the nest cavity, was analysed in their study. Similarly, this study found a correlation between the volume of the acorn and the colony size. During another study, *T. crassispinus* colonies inhabited more frequently bigger artificial cavities, when pieces of wood with different volume cavities were available (Mitrus 2015). In this study, no difference in the volume of the acorns with ant colonies and those containing no ants was found. However, the volume of the acorn itself may not be directly connected with the available chamber for the ants; additionally, the sample sizes were small.

Estimating ant colony size is important for many studies, but typically is not easy, and used method could have strong influence on colonies. Such methods like nest excavation or opening nest chambers are destructive. The mark-release-recapture method is time-consuming – it involves capturing, marking, and recapturing of individual workers (Skorka et al. 2006, Chen & Robinson 2013). Additionally, as in colonies of ants of the genus *Temnothorax*, a large proportion of workers could not be active for longer periods (Charbonneau & Dornhaus 2015), such methods are probably not suitable for the ants. For *Myrmica* species, fast and non-destructive methods, based on a tendency of workers to climb up on wooden sticks put into their nests, were described (Skorka et al. 2006). This method gives an average estimate of about ±40% of real nest size when based on a single sample, thus may be enough during a study on ants living in large colonies. Probably, this method can be used not only for *Myrmica*, but also for aggressive ant species like genera *Lasius* and *Formica* (Skorka et al. 2006), but not for *Temnothorax* species. Generally, methods should be tested for specific species or groups of similar species – for example the study by Ballard & Pruess (1980) used method that provided good estimates for some colonies and species but unsatisfactory estimates for others.

The described in this paper method could help find if an ant colony inhabits an acorn without destroying it, but does not provide sufficient means to estimate the exact size of the colony inside. Another possibility to determine if in any potential nest sites for ant colonies are present – like acorns or twigs – is just through the direct observation of such objects. During such observations, it is often possible to notice workers, e.g., on an acorn containing a colony

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or just emerging from a nest cavity. Still, such a potential site will need to be observed longer, and typically only one or two workers can be simultaneously observed. Thus, the direct observation of potential nest sites in the field is not useful when looking for ant colonies on a larger scale. Placing traps with honey in the field could help find the presence of acorn ants in a specific area (through personal observation). Still, it is not easy to find the nest sites, as the maximum observed foraging distance of the workers of *T. crassispinus* could be larger than 1.5 m (Fokuhl et al. 2012). A small arena – in the study, a 10.2 cm × 10.2 cm Petri dish was used – enabled all the workers outside the nest cavity to be easily observed. Additionally, keeping the potential sites at a low temperature (in the study, 7°C, for more than 24 hours) and then carrying them to a much higher temperature (22°C) probably stimulated the workers to start foraging. Thus, the described method could be useful for studies in which information about the presence of an ant colony in exact potential nest sites is important, but the nest sites should not be destroyed.

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Streszczenie

Informacje dotyczące lokalizacji gniazd mrówek oraz możliwość oszacowania liczebności kolonii bez otwierania gniazd mogą być przydatne w trakcie wielu badań. W niemniejszej pracy opisano próbę oszacowania wielkości kolonii mrówek z rodzaju *Temnothorax* zamieszkujących żołędzie bez otwierania i niszczenia potencjalnych miejsc gniazdowych. W trakcie badań, we wrześniu i październiku, zebrano żołędzie i przeniesiono je do laboratorium. Następnie po ponad 24 godzinach przetrzymywania w lodówce, każdy żołądź umieszczano w osobnej szalce Petriego, w której dostępny był pokarm (miód) i prowadzono obserwacje przez 90 minut. Policzono robotnice mrówek widoczne na zewnątrz żołędzi. Następnie otworzono żołędzie i policzono znajdujące się w nim mrówki. Metoda okazała się skuteczna w rozróżnieniu, w których z zebranych żołędzi znajdowały się kolonie mrówek, ale bez otwarcia gniazda nie było możliwości oszacowani rzeczywistej wielkości kolonii. Opisana metoda może być przydatna w badaniach, w których istotna jest informacja o obecności kolonii mrówek w potencjalnych miejscach gniazdowych.

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