Biologia (2022) 77:125–135
https://doi.org/10.1007/s11756-021-00901-y

ORIGINAL ARTICLE

Laboratory observations on *Anergates atratulus* (Schenck, 1852): mating behaviour, incorporation into host colonies, and competition with *Strongylognathus testaceus* (Schenck, 1852)

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Received: 24 February 2021 / Accepted: 25 August 2021 / Published online: 27 October 2021

Abstract

Uncovering the world of social parasitic ants is difficult due to their rarity and local distribution. Moreover, the more advanced the stage of parasitism, the rarer the species. *Anergates atratulus* (Schenck, 1852) is an extreme workerless parasite that lives with host species of the genus *Tetramorium* Mayr, 1855 and is locally distributed throughout large parts of the ranges of its hosts. In this study, two queenless colony fragments of *A. atratulus* and two of the degenerate slave-raider*Strongylognathus testaceus* (Schenck, 1852) were collected in the field and kept in a laboratory. The host species, *Tetramorium caespitum* (Linnaeus, 1758) and *T. staerckei* (Kratochvíl, 1944), were identified in an integrative fashion combining worker morphometrics with COI gene identification and revealed *T. staerckei* as a new host species of *A. atratulus.* Novel sexual and host-parasitic behaviour was observed: Gynes of *A. atratulus* successfully obtained from breeding in laboratory nests exhibit female-female competition behaviour for males. Ten incorporation attempts to queenless mixed colony fragments of *T. caespitum* and *S. testaceus* were conducted. The workers of *T. caespitum* and *S. testaceus* killed all gynes of *A. atratulus*.

Keywords Social parasitism · Inquilinism · Degenerate slave-raiders · Female-female competition · *Tetramorium caespitum* complex · Morphometrics · COI gene

Introduction

Social parasitism, one of the most remarkable phenomena in the world of ants, has fascinated biologists for more than 200 years (e.g., Huber 1810; Darwin 1859; Fabre 1879; Lubbock 1883; Wasmann 1908; Wheeler 1910). In this relationship the survival of one social species (i.e., the parasite) depends on one or several free-living social species (i.e., the host). There are several comprehensive overviews of social parasitism in ants (Kutter 1968; Hölldobler and Wilson 1990; D'Ettorre and Heinze 2001; Buschinger 2009; de la Mora et al. 2020). The most emblematic type called "inquilinism" represents permanent parasitism without slavery, where the host workers take over all husbandries in the nest and the parasitic queen focuses all energy into the production of sexuals. It is considered to be the most derived type of social parasitism (Buschinger 2009). One typical representative is *Anergates atratulus* (Schenck, 1852). This rarely collected social parasite (Buschinger 1999; Buschinger et al. 2003) of species of the genus *Tetramorium* has completely lost the worker caste and is presented only by gynes and a pupoid type of males. The mating occurs inside the nest of the host species and later, young winged gynes disperse by flying. After losing their wings they penetrate queenless colonies of *Tetramorium*(e.g., Adlerz 1886; Wasmann 1908; Wheeler 1910; Crawley 1912; Crawley and Donisthorpe 1913; Buschinger 1999; Buschinger 2009; Seifert 2018). *Anergates atratulus* occurs locally in large parts of the ranges of its host species (Czechowski et al. 2012; Seifert 2018).

The other group of social parasites of the genus *Tetramorium* belongs to the Palearctic genus *Strongylognathus* Mayr, 1853, which forms a monophyletic group (Sanetra and Buschinger 2000) of about 25 species, all of them characteristically equipped with specialized sharp mandibles adapted for piercing the head of their resisting

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victims via the mouth opening (Kutter 1968; Seifert 2018). The majority of these species displays permanent parasitism with slavery (dulosis), a type of relationship in which parasitic species depend upon their hosts throughout their life and replenish their slaves by raiding the brood of *Tetramorium* colonies (Kutter 1968; Buschinger 2009; Sanetra and Güsten 2001). There are also two host-queen-tolerant inquiline species, *Strongylognathus testaceus* (Schenck, 1852) and *Strongylognathus karawajewi* (Pisarski, 1966), which lost the ability to conduct slave raids. In these cases, *Strongylognathus* gynes penetrate the host nest without killing the queen and then both produce offspring. The parasitic queen suppresses the host species' production of sexuals by pheromonal inhibition (Buschinger 1999, 2009; Seifert 2018). In strongly populated nests the *S. testaceus* workers usually do not exceed 5 % of the workers of the colony (Czechowski et al. 2012; Seifert 2018).

Ants of the genus *Tetramorium* are one of the most difficult genera to determine on species level due to great variability of workers in both morphometrics and body sculpture. Many cryptic species were resolved recently (Schlick-Steiner et al. 2006; Csősz and Schulz 2010; Wagner et al. 2017). Resolving the taxonomy of the complicated *Tetramorium caespitum* complex has made the knowledge about the host preferences of its social parasites less clear (Tinaut et al. 2005; Lapeva-Gjonova et al. 2012). There are six confirmed host species of *A. atratulus*: *T. caespitum*, *T. chefketi* Forel, 1911, *T. diomedeum* Emery, 1908, *T. immigrans* Santschi, 1927, *T. impurum* (Foerster, 1850), and *T. moravicum* Kratochvíl, 1941 (Sanetra et al. 1999; Lapeva-Gjonova et al. 2012; Wagner et al. 2017; Seifert 2018). Determination of the hosts of *S. testaceus* resulted in four species: *T. alpestre* Steiner, Schlick-Steiner & Seifert, 2010, *T. brevicorne* Bondroit, 1918, *T. caespitum*, and *T. impurum*(Sanetra et al. 1999; Wagner et al. 2017; Seifert 2018). Ward et al. (2015) proposed a synonymisation of *Anergates* under *Tetramorium*. However, since this topic is still being discussed within the myrmecological community (Seifert et al. 2016; Ward et al. 2016; Kiran et al. 2017; Werner et al. 2018; Seifert 2018), we follow the nomenclature of Seifert (2018).

This research was the opportunity to create a mixed colony using both of these rarely collected parasites, *A. atratulus* and *S. testaceus*, with its host species and test the potential of its co-existence under laboratory conditions. Our suggestion is supported by one finding of a mixed colony of *A. atratulus* with a different member of the genus *Strongylognathus*, that is, *Strongylognathus alpinus* (Wheeler, 1909) and its host species (Seifert 2018). We focused on testing this hypothesis by observing the 10 penetration attempts of young mated gynes of *A. atratulus*, which we successfully obtained from our own breeding in laboratory nests. Simultaneously, we described several noteworthy observations from the mating behaviour of this rare species.

Material and methods

Sampling

The fieldwork was conducted on 22nd April 2019 on inland sand dunes in the Military Training Area Záhorie (Slovakia; 48°32'26" N, 17°15'17" E; 218 m a.s.l.) – a part of Natura 2000 network which preserves European natural heritage. The sample plot (100 × 50 m) was a typical lowland sandy grassland with *Corynephorion canescentis* Klika, 1931 association (Fig. 1). Searching for potential host nests consisted of finding nest entrances and uncovering the upper layer of sand to expose the ants. The searching method depended on inspecting the brood of ants, where according to current scientific knowledge, *Tetramorium* nests associated with *A. atratulus* or *S. testaceus* do not contain larvae of alates of the host (Czechowski et al. 2012; Seifert 2018). In the majority of nests, the large larvae of *Tetramorium* alates were already present at the study plot (Fig. 2). Nests without such larvae were investigated with greater effort and led to the detection of two nests each with *A. atratulus* and *S. testaceus*. Brood of *Anergates* and *Tetramorium* was easily identified by the difference in their colour; though under strong sunlight both appear similarly white in colour and can be misleading (see Heinze et al. 2007). Simultaneously, two nests of *S. testaceus* were detected by the presence of its workers. Individuals of *A. atratulus* and *Tetramorium* sp. were collected (leg. & det. A. Purkart) and stored in absolute ethanol for taking photos. Moreover, a part of parasitised nests was carefully removed with living material of *A. atratulus* or *S. testaceus* into a transport container (leg. & det. A. Purkart) for laboratory observations. *Tetramorium* workers of the four nests associated with parasites were also collected and stored in absolute ethanol for morphometric and molecular analyses.

Identification of host species

Two colony fragments, termed T_A1 and T_A2, were associated with *A. atratulus*, two further colony fragments, termed T_S1, T_S2, with *S. testaceus*. Host workers of the four parasitized colonies were identified in an integrative fashion combining two independent methods, that is, worker morphometrics and COI gene identification.

Morphometric data were collected using LEICA MZ16 A - high-performance stereomicroscope with a magnification range of 80–294×. Data of 31 characters of one worker per nest were taken to, primarily, use the key of Wagner et al. (2017). Following the methods presented by Seifert et al. (2013), a 10-class linear discriminant analysis using morphometric data from Table S6 in Wagner et al. (2017) as a calibration set and morphometric data of the four workers of this study as wild-cards was additionally performed in SPSS Statistics v16.0 (IBM, USA).

Fig. 1 Sampling plot on sand dune in Military Training Area Záhorie in Slovakia (photo: A. Purkart)

From each parasitized nest two *Tetramorium* workers were used (i.e., T_A1_3, T_A1_4; T_A2_2, T_A2_3; T_S1_2, T_S1_3; T_S2_2, T_S2_3) for COI gene identification. Genomic DNA was extracted from gaster of workers by E.Z.N.A.® Tissue DNA kit (OMEGA bio-tek) according to the manufacturer's protocol. DNA is stored at -25 °C at the Department of Zoology of Faculty of Natural Sciences, Comenius University in Bratislava (Slovakia). A part of the cytochrome oxidase subunit 1 (COI) gene, that is, a fragment of the mtDNA, was used. A 1031 bp fragment was amplified using the primers COI1f: 5'-CCC CCC TCT ATT AGA TTA TTA TT-3' (Steiner et al. 2005) and UEA10: 5'-TCC AAT GCA CTA ATC TGC CAT ATT A-3' (Lunt et al. 1996). Standard PCR was performed using by DreamTaq™ Green DNA Polymerase (Thermo Scientific) for a total volume of 25 µl, comprising 100–200 ng genomic DNA, 2.5 µl DreamTaq™ Buffer, 2.5 µl 25 mM $MgCl_2$, 2 µl of dNTP Mix, 1 µl of 3.0 pmol/ml each primer, 0.4 µl (5 U/µl) DreamTaq™ DNA polymerase and nuclease-freewater to 25 µl. Reactions were carried out on an Eppendorf thermal cycler with in initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, 48 °C for 45 s, and 72 °C for 2 min, and 10 min of final extension at 72 °C. All PCR products were detected on 1 % agarose gel stained with GoldView. Purification and Sanger sequencing were performed in a commercial laboratory, Macrogen Europe Inc. (Amsterdam, The Netherlands) using both amplification primers. COI gene sequences were compared with sequences published by Wagner et al. (2017) available at the homepage of the National Center for Biotechnology Information (USA, https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Highest percent identity values were accepted as COI-gene cluster identification.

Laboratory observation

The experiments started 25 days after sampling in the field (17th May 2019) and ended on 19th June 2019. All ant colony fragments were kept in laboratory nests made of plastic, gypsum and sand, where transparent coverage of nest chambers allowed detailed observations. The nests consisted of three horizontal chambers with a size of 30 × 30 × 5 millimetres with a single outlet to the arena (plastic tank 30 × 20 × 12 centimetres), which represented the outside world. All the nests were placed in a laboratory (air temperature approx. 22 °C) with a constant low light, which provided proper all day observations. Ants were fed with sugared water (1 part sugar with 3 parts water) and solid foods (dead *Drosophila melanogaster* Meigen, 1830) every second day. Water was provided *ad libitum*. Observations of sexual and host-parasitic behaviour were conducted in laboratory conditions in two steps:

1) Sexual and social parasitic behaviour: Two colony fragments each with 250 *Tetramorium* workers and 120 *A. atratulus* pupae (100 gynes, 20 males) were used. The numerical female-biased sex ratio 0.83 (gynes / total sexuals) followed the results of Heinze et al. (2007). The duration of copulation was measured in ten randomly selected pairs.

Fig. 2 I: The development stages of *Tetramorium* cf. *caespitum* brood: **a** worker larva, **b** worker mature larva, **c** worker pre-pupa, **d** alate mature larva; II: The development stages of *Anergates atratulus* brood: **a** alate mature larva, **b** alate pre-pupa, **c** gyne pupa dorsal view, **d** gyne pupa ventral view. All specimens were collected on 22th April 2019 at one sampling plot (photos: D. Selnekovič)

2) *Anergates-atratulus*-gyne incorporation observations: Ten mated gynes of *A. atratulus*, which left their host colony fragment (five of each) and lost their wings, were put one by one next to the entrance of the two mixed colony fragments with 250 *Tetramorium* workers and 10 *S. testaceus* workers and their brood. The demography 4) % of all workers belonged to *S. testaceus*) of the simulated orphaned colony fragments followed the data of Seifert (2018). In total, five incorporation observations using a single gyne to each nest in three days intervals (10 cases total) were provided.

To capture the observations, a Canon EOS 80D body with a Canon MP-E 65-mm f/2.8 1–5 × Macro Lens (Canon, Tokyo, Japan) and a Levenhuk DTX 50 Digital Microscope were used. Dead specimens were picked off and stored in 96 % ethanol. Digital photographs of larvae and pre-pupae were taken using a Canon EOS 5D Mark II camera attached to Zeiss Axio Zoom.V16 stereoscope using temporary preparation. Image stacks were produced manually, combined using Zerene Stacker software and edited in Adobe Photoshop CC. A standard stopwatch was used to measure the time of all presented observations.

Results

Identification of host ant species

Morphometric discriminate results combined with geographic information using a key (Wagner et al. 2017): Sample T_A1 was identified as *T. staerckei*, samples T_A2, T_S1, and T_S2 as *T. caespitum.*

A wild-card run of morphometric data in a 10-class linear discriminant analysis including data of workers investigated by integrative taxonomy in Wagner et al. (2017) assigned the single worker of sample T_A1 with p = 1 to *T. staerckei*, those of T_A2, T_S1, and T_S2 with p = 1, 0.87, and 0.93, respectively, to *T. caespitum*.

The fragments of the COI genes of *Tetramorium* workers belong to COI clusters of the species *T. staerckei* (T_A1, 99.52 % identity) and *T. caespitum* (99.81, 99.71, and 99.90 % identity, respectively). These COI clusters had been identified in an integrative taxonomic approach (Wagner et al. 2017). The four new haplotypes (T_A1, T_A2, T_S1, T_S2) are available in GenBank under numbers MN602021-MN602028.

Since all morphological identifications following the key of Wagner et al. (2017) and doing the wild-card analysis were in concordance with those of the COI-gene identification, an unambiguous identification result of host species can be presented: Colony T_A1 belongs to *T. staerckei*, the colonies T_A2, T_S1, and T_S2 belong to *T. caespitum*.

Laboratory observation

Sexual and social parasitic behaviour of *A. atratulus* in *Tetramorium* nests

In both laboratory nests composed of *A. atratulus* brood associated with its host species, the *A. atratulus* gynes developed almost a day earlier than males. When the first adult male of *A. atratulus* emerged, it was ready for mating several hours later. Mating behaviour of *A. atratulus* usually started when one or several gynes began licking the prominent genitalia of a male. Furthermore, gynes exposed their abdomen to the males and tried to move backward so that the male was able to climb on its back and grab it. When the copulation itself started, the other gynes were licking and biting the connection of the mating pair (Fig. 3). Mating disturbance behaviour gradually disappeared, when more males became available. In an undisturbed position both mating sexuals were immobile and the gyne put all six legs around the connection with the male (Fig. 4). They were carried many times by workers in this position similarly as pupae. Measuring the duration of copulation in 10 random chosen pairs resulted in 98 ± 30 (min = 42, max = 145) minutes. Mating behaviour took place continuously during the day and night. The feeding of *A. atratulus* alates by host workers was not observed. When no adult gynes were available, males tried to copulate with gyne pupae (Fig. 5). Mated gynes seemed to be no more interested in males and, usually, left the nest several hours after mating or were gently carried out of the nest by host workers. Flying or any kind of simultaneous nest leaving of gynes was not observed. Later, they dropped the wings using their hind legs and died within two days. Similarly, all gynes that hatched at a time when the males were no available died at the same interval. Only one mated *A. atratulus* gyne stayed in a laboratory nest and reached physogastrism without shedding its wings (Fig. 6). It did not produce any eggs and died within three weeks.

Introduction of *A. atratulus* gynes to *S. testaceus* nests

All cases of experimental incorporations started with the dealate *A. atratulus* gynes walking around the area close to the potential host-nest entrance, until they were perceived by foraging workers. Workers attacked them, but the aggression stopped after a few seconds (Fig. 7). Then it walked itself or was dragged by the *Tetramorium* workers inside the nest, while it was continuously licked. The course of transport was not continuous, but was interrupted by the occasional dropping of females on the ground and re-lifting. Inside the nest, workers of *Tetramorium* and *S. testaceus* were observed cleaning its body, mostly on the tip of the abdomen. The crucial phase for gynes started in seven cases about 30 min after the penetration, when first *Tetramorium* workers, later with a help of *S. testaceus* workers, evoked to bite a body appendages of the gynes of *A. atratulus*, what caused their death around one hour later. In the remaining three cases, the test appeared successful for the first hour of the experiment, where all gynes were walking freely inside the brood chambers of the host nest (Fig. 8). In these cases, when they got almost no attention from the *Tetramorium* workers, *S. testaceus* workers killed all three gynes of *A. atratulus* within the next hour by penetrating their cuticula on the head and thorax (Fig. 9). Thus, in all ten cases the establishment of stable mixed colonies was unsuccessful.

Discussion

Sampling method of *A. atratulus* and *S. testaceus*

Studying the ant assemblages of Central European sand dunes offered the great chance to study covered ant species. Identification of *A. atratulus* brood by its grey colour, as mentioned by Heinze et al. (2007), might not be much useful under strong sunlight, where its colour appeared similar white as the brood of *Tetramorium* workers. Thus, identification primarily using the appearance of pupae (size, gyne wing pads, bulging ocellar area) is recommended at the season when both species have brood. We perceived that, while sexuals of *Tetramorium* cf. *caespitum* were at the larval stage on the 22nd April 2019, those of *A. atratulus* were already developed as pupae; this phenological difference is already known (Seifert 2018).

Fig. 3 Two gynes of *Anergates atratulus* lick and bite the connection of copulating pair of the same species (photo: A. Purkart)

Identification of host ant species

Our results extend the knowledge of host preferences of social parasites: While *S. testaceus* was found only in nests with *T. caespitum*, we present the first data of an association between *A. atratulus* and *T. staerckei*. In addition with the so-far known host species, seven host species of *A. atratulus* are currently confirmed.

New knowledge about sexual behaviour of *A. atratulus*

While unmated gynes of *A. atratulus* disturbed the copulation via biting, copulating gynes put all six legs around the connection to fix the mating position. We interpret both as adaptations in a female-female competition for the access to males: While the disturbing strategy might be adaptive to potentially

Fig. 4 Mating of *Anergates atratulus* in immobile stage with all female's legs put on the male's genitalia (photo: A. Purkart)

Fig. 5 *Anergates atratulus*: Mating attempt of the male with female pupa (photo: A. Purkart)

split a mating pair (which was not observed) for increasing the chance to copulate with the male, the defence strategy against unmated gynes via fixing the mating position might minimize the costs of an interruption during the copulation. Different strategies of ergatoid male-male competition inside the nest are known among ants (Hamilton 1979; Heinze et al. 1993; Yamauchi et al. 1996; Jacobs and Schrempf 2017), but similar female-female competition was unknown. Generally, the observed female competition for mating – if our interpretation is right – is remarkable, since in most animal cases the male sex competes for mating (Wilson 1975; Voland 2009). As presented by Rosvall (2011), if the operational sex ratio becomes more female-biased, females should compete for the access to males. In our laboratory nests we observed female-female competition only after the first adult males of *A. atratulus* emerged. The behaviour had gradually decreased with the increasing number of available males in the nest. Previous authors (e.g., Adlerz 1886; Wasmann 1891; Wheeler 1910; Heinze et al. 2007) did not recognize the female-female competition behaviour during their observations – it seems too elusive or larger laboratory test numbers might be needed for such observations. Nevertheless, the

Fig. 6 Physogastric alate *Anergates atratulus* gyne which avoided leaving its host nest after mating (photo A. Purkart)

Fig. 7 *Tetramorium* workers perceive the *Anergates atratulus* gyne after its incorporation into the nest (photo A. Purkart)

ultimate background behind the female-female competition behaviour should be investigated in detail. During the research, copulation attempt between males and gyne pupae was observed. On the contrary, this behaviour could be the result of competition between males trying to be among the first near freshly hatched gynes. The duration of copulation observed in our laboratory nests was 98 ± 30 (min = 42, max = 145) minutes. With maximal duration of 145 min, *A. atratulus* is the ant species with the longest lasting copulation ever known. This is in line with the results of Heinze et al. (2007), who mentioned the time exceeding one hour, however, not corresponding with Boer (2015) who reported just 5–15 min. It is still uncertain, whether *A. atratulus* gynes mate with one or several males, but their leaving of the colony several hours after the first mating indicates the option of single mating. Moreover, no feeding of *A. atratulus* gynes

Fig. 8 *Anergates atratulus* dealate gyne moving freely in *Tetramorium caespitum* nest associated with the social parasite *Strongylognathus testaceus* (photo: A. Purkart)

Fig. 9 *Strongylognathus testaceus* workers kill the *Anergates atratulus* gyne (photo: A. Purkart)

by *Tetramorium* host workers was observed. Similarly, Adlerz (1886) and Wasmann (1891) noticed only little attention to virgin *A. atratulus* gynes by *Tetramorium* workers in their artificial setups. In one of our laboratory nests, one mated but alate *A. atratulus* gyne stayed in a laboratory nest and reached physogastrism but did not produce any eggs and died after 3 weeks. Similar observations were provided by Crawley (1912), who successfully performed an experimental incorporation of an *A. atratulus* gyne into a *Tetramorium* colony, but it also died without having laid any eggs. Other gynes of our study, which left the laboratory nest to outside world, lost their wings using their hind legs, never came back to the host nest and died soon. While simulation of swarming behaviour in other species is difficult (Jacobs and Schrempf 2017), an intranidal mating of *A. atratulus* under laboratory conditions confirmed this species as available subject for indoor observation of ants mating. In return, studying ant mating behaviour merely under laboratory conditions might not fully uncover the entire spectrum of behaviours that are affected. Additionally, specific conditions prevailing in the sand dune environment where colony fragments were obtained (i.e. artificial photoperiod, thermal changes, and unknown food sources) combined with changes of its social structure may also take a role in the results of this study.

Incorporation of *A. atratulus* gynes

Based on the experiments of Adlerz (1886), Wasmann (1891, 1908), Wheeler (1910), and Crawley (1912), young *A. atratulus* gynes were introduced to a queenless host colony of the *T. caespitum* complex. In this study, the introduction of parasitic gynes was unsuccessful. In seven of ten cases *Tetramorium* workers killed them. We noticed about a half-hour delay between the start of the incorporation attempt and the killing of social parasite gynes. In the world of ants, social parasites use two different methods of integrating into a host colony: 'chemical camouflage', where the parasite acquires the colony odour from the host species or the nest surroundings by allogrooming, or 'chemical mimicry', where the intrinsic biosynthesis of host cuticular hydrocarbons by the parasite actively reproduces the host's odour profile (Dettner and Liepert 1994). Our study did not provide enough information to assume which method prevailed in this case and further research is needed to explain this phenomenon. Following the results of this experiment, a similar situation in nature would at least partly explain the rarity (Wheeler 1910; Buschinger et al. 2003) and low nest densities (Seifert 2017) of *A. atratulus*. In three cases, the *A. atratulus* gynes successfully invaded the mixed colony fragment. After a short time of tolerance of three species occurring in the nest, the *A. atratulus* gyne was gently licked (see Seifert 2018) by *Tetramorium* and *S. testaceus* workers, however, no feeding was noticed. These facts combined with aggression of *S. testaceus* workers against *A. atratulus* gynes reject the assumption of composing mixed colonies. The major outcome of this study is thus demonstrating the *Strongylognathus testaceus* as a stronger interspecies competitor than *A. atratulus*. The observation of aggression in *S. testaceus* workers raises the question if the production of workers in *S. testaceus* might be still useful. Losing the ability of conducting raids and the process of reducing the number of workers in the colony was considered as step to evolve to an inquiline (Kutter

1968; Hölldobler and Wilson 1990; Sanetra and Buschinger 2000; Tinaut et al. 2005). According to our observations, the *S. testaceus* workers killed *A. atratulus* gynes by penetrating into the head and thorax with their mandibles. It should be proven if their defensive power and killing methods are adaptive against the other special invaders which are not eliminated by *Tetramorium* workers. This result also extends the hypothesis that S. testaceus workers may actively suppress the host sexual production (Seifert 2018). Although our research has not ruled out the possibility that a mixed nest of a host species and two social parasites is possible, it is likely that, if this situation occurs in nature, it is extremely rare. Either way, there may be several reasons why this experiment was not successful in the laboratory conditions, that is small number of repetitions and reusing the same queenless colony fragments. The live study material used in this research is very difficult to obtain in the field survey due its rarity, which made it almost impossible to perform a larger number of repetitions of experiments. It will therefore be necessary to examine the individual observations in more detail in future research.

Acknowledgements Bernhard Seifert provided the microscope used for morphometrics in this study. The research was financially supported by Scientific Grant Agency of Ministry of Education and Sports and Slovak Academy of Sciences (VEGA 2/0032/19, 1/0007/21) and Slovak Research and Development Agency (grant number APVV-15-0147).

Author contributions All authors contributed to the study conception and design, material preparation, and writing of the manuscript. Data collection and analysis were performed by Adrián Purkart and Herbert C. Wagner. All authors read and approved the final manuscript.

Funding The research was financially supported by Scientific Grant Agency of Ministry of Education and Sports and Slovak Academy of Sciences (VEGA 2/0032/19, 1/0007/21) and Slovak Research and Development Agency (grant number APVV-15-0147).

Data availability All material in current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Consent to participate Not applicable.

Consent for publication Not applicable.

Conflicts of interest/Competing interests The authors declare that they have no competing interests.

Ethics approval All applicable international and national guidelines for the care and use of animals were followed.

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