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Temporal variation in social structure and worker reproduction in the temporary social parasite *Lasius fuliginosus* (Hymenoptera: Formicidae)

Tobias van Elst & Jürgen Gadau

Abstract

Ant societies exhibit striking diversity in their social systems, including variation in the number of queens and mating partners. Knowledge on the number of breeders in a colony is crucial for a better understanding of the evolution of social insect life history traits such as reproductive skew or worker reproduction. Little is known about the breeding system of the formicine ant *Lasius fuliginosus* (Latreille, 1798), even though it is widely distributed in the Palearctic and able to compete ecologically with dominant genera like *Formica*. Moreover, *L. fuliginosus* has a particularly interesting life history in that it is a temporary social parasite of several *Lasius* species, which themselves are temporary social parasites. We determined the number of (reproductive) queens and mating partners of *L. fuliginosus* colonies and queens, respectively, from a population in Münster, Germany. Workers from 33 colonies and males from 12 of these colonies were genotyped for four polymorphic microsatellite markers. Our results show that 29 of these colonies were monogynous and monandrous and that two colonies were monogynous and polyandrous. Workers of the remaining two colonies were derived from multiple queens, possibly due to adoption of unrelated queens after the original queen's death. Furthermore, genotyping of male offspring provided evidence for worker reproduction in three colonies, potentially also in response to queen orphanage in two of these. We estimated the mutation rate at one microsatellite locus in *L. fuliginosus* to be 1.46×10^{-3} mutations per generation, which is similar to what has been observed in *Apis mellifera* Linnaeus, 1758 and *Drosophila melanogaster* Meigen, 1830. To our knowledge, this is the first study to provide molecular insights into the breeding system of *L. fuliginosus*, which appears to be characterized by facultative polyandry and monogyny. In addition, *L. fuliginosus* now represents the second species in the genus *Lasius* for which worker reproduction has been documented.

Key words: Ants, *Lasius*, microsatellites, genetic structure, worker reproduction, facultative polyandry, monogyny, queen adoption, mutation rate.

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Tobias van Elst (contact author) & Jürgen Gadau, Institute for Evolution and Biodiversity, University of Münster, Hüfferstraße 1, 48149 Münster, Germany. E-mail: t_vane02@uni-muenster.de

Introduction

Ants exhibit great intra- and interspecific diversity in their social systems (Hölldobler & Wilson 1977, 1990, Bourke & Franks 1995). An important feature of ant social systems is the breeding system, which includes the number of breeders (female and male), their relatedness, and reproductive skew between breeders (Goodisman & Hahn 2005). The breeding system determines the sociogenetic structure of a colony (Ross 2001) and is associated with several life history traits (e.g., worker reproduction) that likely shape evolutionary processes (Keller 1993, Bourke & Franks 1995, Ross 2001). Therefore, the evolution of life history traits in ant species can only be understood with knowledge of the breeding system.

The ancestral state of the ant breeding system is thought to be monogyny and monandry, i.e., a colony is headed by one singly mated queen (Hughes & al. 2008, Boomsma & Gawne 2018). Therefore, multiple-queen societies (polygyny) or multiple mating by queens (polyandry) represent derived states. The evolution of multiple breeders is still not fully understood. It poses a potential conceptual problem to kin selection theory (Hamilton 1964) because the presence of multiple breeders in a colony dilutes the relatedness among workers and hence also reduces the inclusive fitness advantage that workers obtain from rearing their siblings. Certain ecological advantages associated with polygyny and polyandry must clearly have facilitated the secondary evolution of multiple breeders in a colony.

Polygyny has evolved many times (Hölldobler & Wilson 1977, Bourke & Franks 1995) among ant social systems. Likely, the most common route to polygyny is queen adoption, where mature and queenright colonies adopt newly inseminated queens after their mating flight. This so-called secondary polygyny might be favourable if dispersal costs are high (Bourke & Franks 1995). An alternative route to polygyny is through pleometrosis. That is, several newly mated queens found a colony together. Such foundress associations can evolve when increased group survivorship outweighs the disadvantage of sharing reproduction in a colony (Bourke & Franks 1995). In most cases, all but one queen are killed after the colony founding period, but

in a few species, several queens stay alive to share colony reproduction (primary polygyny) (e.g., Mintzer 1987, Hölldobler & Carlin 1989, Rissing & al. 1989, Overson 2011, Helms & Cahan 2012). Ecological factors promoting polygyny are for example heavy predation (Rosengren 1983, Bolton 1986), habitat patchiness (Rosengren 1983, Bourke & Heinze 1994), or habitat saturation (Herbers 1986). Moreover, Boulay & al. (2014) showed that in 149 Palearctic ant species polygyny was significantly correlated with ecological dominance and larger colony size, suggestive of the potential advantage of polygyny in interspecific competition.

Many ant species show some multiple mating (Bourke & Franks 1995). However, polyandry in ants is mostly facultative (Page & Metcalf 1982, Starr 1984, Hölldobler & Wilson 1990, Keller & Reeve 1994) and only few genera are known to exhibit obligate multiple mating (see Villesen & al. 2002, Gadau & al. 2003, Denny & al. 2004, Kronauer & al. 2004, Pearcy & al. 2004, Rheindt & al. 2004, Wiernasz & al. 2004, Pol & al. 2008). The benefit of multiple mating might include a more diverse workforce, which would be more efficient (Starr 1984, Crozier & Page 1985) and less prone to parasites (Sherman & al. 1988, Schmid-Hempel 1997), a reduction in variance of diploid (sterile) male production (Page 1980, Page & Metcalf 1982, Crozier & Page 1985, Page 1986), and a reduction of kin conflicts over sex allocation between queens and workers (Starr 1984, Woyciechowski & Łomnicki 1987, Sundström 1993). Costs and benefits of multiple mating in ants were reviewed by Baer (2016).

When studying ant breeding systems, it should be considered that in many species workers represent potential breeders as well. The production of reproductive offspring by workers remains possible if the worker caste has functional ovaries. Since worker-laid eggs are generally not fertilized, worker reproduction is restricted to producing male offspring. In fact, workers are reported to have totally lost functional ovaries only in nine out of over approximately 300 ant genera (Oster & Wilson 1979, Hölldobler & Wilson 1990, Villet & al. 1991) and there is evidence for worker reproduction in more than 40 ant species across 23 genera (Bourke 1988, Choe 1988, Hölldobler & Wilson 1990). However, this number is most likely very conservative since nobody has systematically searched for worker reproduction. Data on more species will be necessary to understand the selective factors that favour and disfavour worker reproduction in ants.

In this study, we provide insights into the breeding system of the formicine ant *Lasius fuliginosus* (Latreille, 1798), which is widely distributed in the Palearctic region (Collingwood 1979, 1982) and shapes local ecosystems due to its territorial and aggressive behaviour and large colonies. *Lasius fuliginosus* populations can have a significant influence on the local assemblage of species in the genera *Formica*, *Lasius* and *Myrmica* (see Savolainen & al. 1989, Czechowski 1999, Czechowski & al. 2013, Markó & al. 2013, Ślipiński & al. 2014). Moreover, *L. fuliginosus* has a particularly interesting life history as a temporary social "hyperparasite". That is, it is a temporary social parasite of several species in the subgenus *Chthonolasius* which also found their colonies by temporary social parasitism (Collingwood 1982, Seifert 2007, Markó & al. 2013). *Lasius fuliginosus* is reportedly polygynous (Donisthorpe 1915, Mattheis 2003, Seifert 2007) alongside only two other species in the genus *Lasius* (see Yamauchi & al. 1981, van Loon & al. 1990). However, the origin of this hypothesis is uncertain and molecular evidence for it is still lacking.

To get a better understanding of the breeding system of *Lasius fuliginosus*, we determined queen numbers and mating frequencies for 33 colonies in a German *L. fuliginosus* population by genotyping workers at four highly polymorphic microsatellite markers. Moreover, males of twelve colonies were genotyped to test whether worker reproduction occurs in this species. This is the first study to provide molecular insights into the breeding system of *L. fuliginosus*.

Material and methods

Sampling: Around 30 *Lasius fuliginosus* workers were hand-sampled from the nest entrance of a total of 33 colonies between June and July 2016 and between April and June 2017 in the vicinity of Münster, Germany. Two colonies were sampled in both 2016 and 2017 (see Tab. S1 in Appendix, as digital supplementary material to this article, at the journal's web pages). Furthermore, males were sampled from the nest entrance of twelve of these colonies as shown in Table S1. The samples were preserved in 100% ethanol. The geographic distribution of the colonies is shown in Figure S1.

Ant species identification: Ants were identified morphologically using the key to European ant species by Seifert (2007). Voucher specimens are reposited at the zoological collection of the Westphalian Museum of Natural History (WMNZ).

DNA extraction: A standard Chelex extraction protocol was used for the extraction of genomic DNA (Gadau 2009). The gasters of females were removed prior to DNA extraction. The specimens were placed into tubes with 100 µl 1 × TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 2 µl Proteinase K (10 mg / ml) and 2 metal beads. After grinding on a Mixer Mill for 20 seconds (28 Hz), 100 µl 10% Chelex in 1 × TE were added. The solution was incubated at 57 °C for one hour followed by a 5 min step at 95 °C. After centrifugation at 14000 rpm for 10 min, 100 µl of the extracted DNA were transferred to a new tube. The extracted DNA was stored at -20 °C.

Microsatellite genotyping: For genotyping, fluorescent-labelled primers for four loci (Lf 03, Lf 04, Lf 05, Lf 06) developed by Falk & al. (2004) were used. To infer the number of queens and mates in the sampled *Lasius fuliginosus* colonies, all four loci were genotyped in approximately 20 workers of each colony, except for colonies Lfu 1 and Lfu 18, where twice as many workers were genotyped. For colonies Lfu 6 and Lfu 8, around 20 workers sampled in both 2016 and 2017 were genotyped. The exact numbers of workers genotyped are given in Table 1.

Polymerase chain reactions (PCRs) contained 1 µl of extracted *Lasius fuliginosus* DNA (diluted 1 : 2.5 with TE buffer), 2.5 mM $MgCl_2$, 1x colourless GoTaq® Flexi buffer, 0.1 mM dNTPs, 4 picomole of each forward and reverse primer and 0.3 U GoTaq® Flexi DNA Polymerase. For each DNA sample one PCR (multiplex) was conducted with primers for the loci Lf 03, Lf 04, Lf 05 at an annealing temperature of 63 °C. Another PCR was conducted with primers for locus Lf 06 at an annealing temperature of 57 °C. The PCRs were carried out on Eppendorf thermocyclers. The initial denaturation step was 94 °C for 3 min, followed by 30 cycles of [40 s at 94 °C, 40 s at the respective annealing

Tab. 1: Sample size and genotyping statistics for the analysis of queen number. Note that queen number refers to the number of queens necessary to explain observed worker genotypes, which might deviate from the number of physically present queens during sampling. *Doubly mated queen.

Colony	No. of workers genotyped	Genotyping failure / missing data				No. of queens
		Lf 03	Lf 04	Lf 05	Lf 06	
Lfu 01	42	0.02	0	0	0	3
Lfu 02	20	0	0	0	0	1
Lfu 03	20	0	0	0	0	1
Lfu 04	20	0	0	0	0	1
Lfu 05	20	0	0	0	0	1
Lfu 06 (2016)	18	0	0	0	0	1
Lfu 06 (2017)	20	0.05	0.15	0.1	0	2
Lfu 07	19	0	0	0	0	1
Lfu 08 (2016)	20	0	0	0	0	1
Lfu 08 (2017)	17	0.41	0.29	0.24	0	1
Lfu 09	19	0	0	0	0	1
Lfu 10	20	0	0	0	0	1
Lfu 11	20	0	0	0	0	1
Lfu 12	19	0	0	0	0	1
Lfu 13	20	0	0	0	0.13	1*
Lfu 14	20	0	0	0	0	1
Lfu 15	20	0	0	0.15	0	1
Lfu 16	19	0.05	0	0.11	0	1
Lfu 17	20	0	0	0	0	1
Lfu 18	40	0.03	0	0	0.05	1
Lfu 19	20	0.05	0	0.05	0	1
Lfu 20	20	0	0	0	0	1
Lfu 21	20	0.05	0	0.1	0	1
Lfu 22	20	0	0	0	0	1
Lfu 23	20	0.05	0	0	0.05	1
Lfu 24	20	0	0	0	0.05	1
Lfu 25	20	0.25	0	0.05	0	1
Lfu 26	20	0.65	0	0.05	0	1
Lfu 27	20	0.15	0	0.05	0.05	2
Lfu 28	20	0.2	0	0.15	0.05	1
Lfu 29	20	0.35	0	0.25	0.05	1
Lfu 30	20	0.05	0	0.05	0	1
Lfu 31	20	0.1	0	0.18	0.05	1
Lfu 32	20	0.6	0.05	0.05	0	1
Lfu 33	20	0.1	0	0.05	0.05	1*
Total	733	0.09	0.01	0.04	0.02	

temperature as stated previously and 40 s at 72 °C]. The final extension step was 72 °C for 5 min.

0.5 µl of both PCR products (multiplex PCR and the PCR for Locus Lf 06), 0.25 µl GeneScan™ - 350 ROX™ size standard and formamide to a total volume of 10 µl were placed together in one well of a 96-well plate so that each well contained the fluorescent-labelled amplified loci Lf 03, Lf 04, Lf 05 and Lf 06 for one DNA sample. After a denaturation step of 5 min at 95 °C and subsequent cooling down to 4 °C, the 96-well plate was placed onto an ABI 3130xl Genetic Analyzer for capillary separation in the POP-7™ polymer by Applied Biosystems™. Scoring was done manually with GeneMapper v4.0 (Applied Biosystems™). For loci that were not amplified, the microsatellite geno-

Tab. 2: Sample size and genotyping statistics for the analysis of worker reproduction. Males were only genotyped for loci at which the queen and its mate did not share a genotype because only then worker reproduction can be identified. Loci for which a queen and its mate shared an allele in a colony are marked with "n.a.". The probability to identify a male as worker-derived in a monogynous and monandrous colony depends on the number of microsatellite loci genotyped (0.5, 0.25, 0.125, 0.0625 for one, two, three and four loci, respectively).

Colony	No. of males genotyped	Genotyping failure / missing data				Worker reproduction
		Lf 03	Lf 04	Lf 05	Lf 06	
Lfu 06	4	0	0	0	0	YES
Lfu 08	31	n.a.	0	0	n.a.	YES
Lfu 09	15	0	0	n.a.	0	NO
Lfu 10	5	0	0	0	n.a.	NO
Lfu 11	8	0.25	0.13	n.a.	0	NO
Lfu 14	20	n.a.	0	0.1	0.1	NO
Lfu 16	20	n.a.	0	0.55	0.1	NO
Lfu 17	16	n.a.	0	0	0	NO
Lfu 19	20	n.a.	0	0	0.05	YES
Lfu 21	11	0.09	0	n.a.	0.18	NO
Lfu 29	20	0.1	0	0.4	0	NO
Lfu 33	20	0	0	0	n.a.	NO
Total	190	0.06	0.01	0.13	0.06	

typing procedure was repeated. In this case, a single PCR was done for each locus at the optimal primer annealing temperature as stated by Falk & al. (2004).

In addition to workers, males from twelve colonies were genotyped to detect worker reproduction. The exact numbers of male specimens genotyped are given in Table 2. Since males are produced parthenogenetically, they cannot inherit alleles of the queen's mate (paternal alleles). However, workers carry the paternal allele and can pass it on to their offspring. Hence, males carrying a paternal allele are strong evidence for worker reproduction. This also implies that worker reproduction is only detectable by means of male genotyping in case the queen and her mate carry different alleles at a given locus because only then males with the paternal allele can be identified. Additionally, since workers carry one maternal and one paternal allele, only half of their offspring will inherit an allele of the queen's mate. Therefore, males were only genotyped for loci for which the queen and her mate showed different alleles (see Tab. 2). This information was inferred from worker genotypes as described in the subsequent section. Males were genotyped for these loci following the protocol for worker genotyping.

Microsatellite data analysis: For population genetic analyses, ten datasets were created by taking the alleles for all four loci of one randomly selected worker of each colony, respectively. Parameters were calculated for each dataset and then averaged (arithmetic mean). In this way bias due to the relatedness of workers belonging to the same colony was avoided.

For each locus, allele frequencies, observed heterozygosity (H_O) and expected heterozygosity/genetic diversity (H_E) (Nei 1973) were calculated from worker genotypes of all sampled colonies. Genepop v4.2 (Raymond & Rousset 1995) was used to test all loci for deviations from Hardy-Weinberg and genotypic linkage equilibrium.

To test for the degree of isolation by distance among colonies, a Mantel test estimating the correlation between genetic distance according to Nei (1987) and spatial distance was conducted with the programme GeneAlEx v6.503 (Peakall & Smouse 2006).

The narrow deduction method of Matesoft v1.0 (Moilanen & al. 2004) was used to infer the most parsimonious number of mating partners from worker genotypes at the loci Lf 03 to Lf 06 for each colony in case worker genotypes could be explained by a single queen. For colonies with worker genotypes that required multiple reproductively active queens, likely matrilines were separated manually and then also tested for the number of mating partners.

The effective paternity was estimated for each polyandrous colony and population-wide according to Boomsma & Ratnieks (1996) and Starr (1984), respectively.

Microsatellite locus quality: A frequent source of error using microsatellite markers are null alleles, which are the result of polymorphisms at the binding sites of microsatellite primers. Such a polymorphism might cause a primer not to bind so that the respective allele would not be amplified during PCR. This can result in misinterpreting heterozygote diploid workers as homozygotes (Jones & Ardren 2003). Consequently, estimates of queen number and mating frequency would be incorrect. A homozygote excess at any locus would be a strong indicator for the presence of null alleles at this locus. We tested for homozygote excess and null allele frequency at each locus using the programme ML-NullFreq (Kalinowski & Taper 2006). No significant homozygote excess was detected at any locus ($P > 0.25$ for each locus, 30000 randomizations). Assuming null alleles were present nonetheless, their frequency was estimated to be extremely low (Lf 03: 0.018; Lf 04: 0; Lf 05: 0.008; Lf 06: 0.004). Therefore, it is unlikely that null alleles affected our estimates of queen number and mating frequency.

Two types of errors can lead to an underestimation of mating frequencies based on microsatellite genotypes of worker offspring. Nonsampling errors occur when a colony contains several patrilines but not a single worker was sampled from one of these because of insufficient sampling sizes. Nondetection errors occur when queen mates possessed identical genotypes so that their offspring would be genetically indistinguishable.

The probability of not detecting a patriline in a set of 20 workers because of nonsampling errors is $(1\text{-}p)^{20}$ where p is the proportion of workers derived from this patriline among workers in the given colony. For example, the probability of a nonsampling error to occur in a set of 20 workers would exceed 0.05 only if one patriline contributed more than 86% to offspring. Such high skew is not likely and even if it was present in the studied population, it would not affect the population-wide effective mating frequency substantially.

The probability of nondetection errors can be calculated as $\Pi\ (\Sigma\ q_i^2)_j$ where q_i describes the allele frequency of the ith allele at the jth locus (Boomsma & Ratnieks 1996). Thus, the probability of nondetection errors is equal to the product of expected homozygosities at each locus. In our dataset the probability of nondetection errors was estimated to be 0.0004. Therefore, we excluded that nonsampling and nondetection errors substantially affected our results.

Results

Mutations: A recent paper by Schlick-Steiner & al. (2015) reported a lack of awareness of recent insertion / deletion (reINDEL) mutations in animal microsatellite studies. We checked our data for mutated alleles and identified two alleles at locus Lf 06 (GenBank acc. no. AY616195) that were only present in a single worker per colony (Tab. 3). In both cases, the allele was lacking one repeat motif compared to another allele that was present in the same colony. Therefore, we suggest a deletion event (loss of one repeat motif) that happened during parental meiosis as the most likely explanation for the observed rare alleles. In total 685 workers (diploid) were successfully genotyped at locus Lf 06. Two mutations at this locus would correspond to a mutation rate of $2 \times (685 \times 2)^{-1} = 1.46 \times 10^{-3}$ mutations per generation. This matches estimations for microsatellite mutation rates in *Apis mellifera* Linnaeus, 1758 (between $\mu = 1.5 \times 10^{-4}$ and $\mu = 1.14 \times 10^{-3}$) by Estoup & al. (1995) and in *Drosophila melanogaster* Meigen, 1830 ($\mu = 3 \times 10^{-4}$) by Schlötterer & al. (1998). We could exclude errors during allele calling and PCR artefacts by re-genotyping the individuals and manual inspecting of the chromatograms. Therefore, both alleles were considered as mutations and were not included in the analysis of queen number and mating frequency. No mutations were identified at the loci Lf 03, Lf 04 and Lf 05.

Tab. 3: Details of identified mutations in two workers for microsatellite locus Lf 06.

Colony	Worker	Sex of allele donor	Ancestral allele	Derived allele
Lfu 18	W25	Male	228	226
Lfu 32	W01	Female	244	242

Statistical considerations: All four loci were highly polymorphic in the studied population with allele numbers ranging from 12 to 15 (for allele frequencies, see Tab. S2). Observed (80 - 92%) and expected (79 - 93%) heterozygosity (Tab. 4) were similarly high to the findings of Falk & al. (2004) for another *Lasius fuliginosus* population. No significant heterozygote deficit ($P > 0.3$ for each locus), excess ($P > 0.1$ for each locus) or genotypic linkage disequilibrium were detected for any locus.

No significant correlation between Nei's genetic (1987) and spatial distance was detected (Mantel test, $r = 0$, $P = 0.497$, 9999 permutations).

Queen number and mating frequency: Queen number and mating frequency were estimated for 33 colonies in total (Tab. 1). Worker genotypes of 29 of the colonies could be explained by one, singly mated queen (monogyny and monandry), as only two or three genotypes were detected per locus (excluding the two mutations, see above) (see Tab. S3 for genotypes). Colonies Lfu 1, Lfu 13, Lfu 27 and Lfu 33 consistently showed genotypes deviating from the monandry / monogyny-pattern.

For colony Lfu 1, the observed worker genotypes could not be explained by polyandry. Hence, it clearly contained workers derived from multiple queens. To explain worker genotypes in this colony with only two reproductively active queens, required for one of these queens to be mated with at least six mates. This seemed not likely regarding the high rate of monandry in the population and the rare occurrence of high mating frequencies in ants in general (Boomsma & Ratnieks 1996). Alternatively, the observed worker genotypes were in agreement with the more likely scenario of three singly inseminated reproductively active queens in the colony.

Matesoft assumes monogyny to explain worker genotypes whenever possible. However, in some cases this assumption is not suitable because it can result in artificially high mating frequencies. For instance, it was possible to explain worker genotypes for colony Lfu 27 with just one queen that mated with seven mates. Again, such amounts of mating partners were unlikely (given the results of the remaining colonies). Two singly mated queens were a more likely explanation for observed worker genotypes (see Tab. S3).

Tab. 4: Number of alleles and observed / expected heterozygosity of four microsatellite loci for the *Lasius fuliginosus* population around Münster, Germany.

Locus	GenBank acc. no.	No. of alleles	H_O	H_E
Lf 03	AY616192	13	0.797	0.785
Lf 04	AY616193	15	0.871	0.815
Lf 05	AY616194	15	0.924	0.926
Lf 06	AY616195	12	0.883	0.861

Our data suggested that colonies Lfu 13 and Lfu 33 were monogynous and polyandrous with two predicted mating partners for each queen. The effective paternities in colonies Lfu 13 and Lfu 33 were $m_e = 1.724$ and $m_e = 1.246$, respectively. In colony Lfu 33 however, a second mating partner carrying an allele "117" at locus Lf 03 was only necessary to explain the observed genotyping pattern because of two workers' genotypes at locus Lf 03 (see Tab. S3). Consequently, the proposed mating partners only differed in the allele "117" at locus Lf 03 but were genetically identical at the loci Lf 04, Lf 05 and Lf 06. This raised the question whether the queen was indeed doubly mated (with high skew between the reproductive contributions of mates making the effective mating frequency almost 1) or whether the allele "117" was a genotyping artefact. The population-wide effective paternity would have been $m_e = 1.017$ assuming double mating due to allele 117 and $m_e = 1.012$ under the assumption that the allele "117" was a genotyping artefact. Therefore, whether the queen of colony Lfu 33 was singly or doubly mated did not have a strong effect on the mode of mating in this *Lasius fuliginosus* population.

In the two colonies Lfu 6 and Lfu 8, workers of two consecutive years were genotyped (see Tab. S4 for genotypes). In colony Lfu 8, worker genotypes found in 2017 were identical to worker genotypes of 2016. Thus, workers of both years were likely derived from the same singly mated queen. In contrast, three workers with new genotypes at each locus were found in 2017 compared to 2016 in colony Lfu 6. These workers could not have been produced by either queen proposed by Matesoft for 2016 since alleles were present in these workers that are absent in both the queen and its mating partner.

In summary, the majority of studied *Lasius fuliginosus* colonies (88%) was monogynous and monandrous. Only two colonies contained workers derived from more than one queen (6%), which were all single mated. Two monogynous colonies contained a doubly mated queen (6%). Hence, polygyny and polyandry did not occur together.

Worker reproduction: To check for worker reproduction in *L. fuliginosus*, we genotyped males for twelve colonies (see Tab. S5 for genotypes). According to our previous findings, all these colonies were monogynous and only colony 33 was not monandrous but was headed by a doubly mated queen. Workers of three of these colonies, i.e., colonies Lfu 6, Lfu 8 and Lfu 19, carried paternal alleles, which is strong evidence for worker reproduction (Tab. 2). Four males were sampled from colony Lfu 6, of which three possessed paternal alleles at the loci Lf 05 and Lf 06. For colony Lfu 8, one male out of 31 possessed paternal alleles at the loci Lf 04 and Lf 05. For colony Lfu 19, 17 of 20 genotyped males possessed a paternal allele at one or more of the loci Lf 04, Lf 05 and Lf 06.

Worker-produced males inherit either the paternal allele or the maternal allele of their worker mother. Hence, only 50% of worker-produced males are expected to carry the paternal allele and can be identified as worker-derived regarding a single locus. Regarding three loci, the probability of a worker-derived male to possess only maternal alleles would be $0.5^3 = 0.125$, so that we would not have been able to identify about two to three males as worker-derived in a set of 20 samples. This fit our data for colony Lfu 19, since three males possessed alleles at all loci that could be either queen- or worker-derived. Therefore, it is quite possible that all genotyped males were worker-derived in colony Lfu 19. A similar calculation for colony Lfu 6 would not be meaningful because only four males were genotyped for this colony. However, the fact that three of four genotyped males were clearly worker-derived also suggests that the majority of males in this colony was produced by workers. Males of colony Lfu 8 could only be genotyped for two loci, so that 0.25 of the samples, i.e., about eight samples, would likely not be identifiable as worker-derived. However, we only found one worker-derived male for this colony. Therefore, it is likely that most males in this colony were produced by the queen.

Discussion

Queen number: Except for *Lasius sakagamii* (Yamauchi & Hayashida, 1970) and the invasive ant *L. neglectus* van Loon, Boomsma & Andrasfalvy, 1990 (Yamauchi & al. 1981, van Loon & al. 1990), all *Lasius* species for which queen number is known are monogynous (e.g., Tanquary 1913, Waloff 1957, Seifert 2007). We found that the majority of colonies in the studied *L. fuliginosus* population were also monogynous. Hence, we could not confirm the previous findings on queen number, i.e., polygyny, in *L. fuliginosus* (see Collingwood 1979, Mattheis 2003, Seifert 2007). This discrepancy can be explained in two ways. First, colonies that contain multiple queens but are effectively monogynous can lead to a difference between molecular (i.e., worker genotyping) and observational data on the number of queens. It is not known what studies the polygyny hypothesis for *L. fuliginosus* is based on since Collingwood (1979), Mattheis (2003) and Seifert (2007) did not provide proper citations for their claims but, most likely, these findings are based on personal observations. Secondly, intraspecific polymorphism of the number of functional queens between populations has been shown for a number of ant species including *L. sakagamii* (see Yamauchi & al. 1981, Ross & Fletcher 1985, Chapuisat & al. 2004, Schlick-Steiner & al. 2007, Gill & al. 2009, Overson 2011, Helms & Cahan 2012). Variation of social organization could therefore also be present among *L. fuliginosus* populations. Such variation might be due to differing environmental conditions, i.e., conditions that favour polygyny (e.g., habitat saturation, predation, inter- / intraspecific competition) are present in one (sub-)population but not in another. Interestingly, variation of queen number can go along with restricted gene flow and genetic differentiation between social forms (e.g., Ross & al. 1997, Gyllenstrand & al. 2005). Considering the conflicting accounts on queen number in *L. fuliginosus*, it will therefore be worthwhile to use molecular methods to estimate the number of queens in colonies of different *L. fuliginosus* populations. It is also worthy of note that Collingwood (1979) and Seifert (2007) reported polydomy for *L. fuliginosus*, which we did not observe in a single colony in the studied population.

Only two of 33 colonies in the studied population, i.e., colonies Lfu 1 and Lfu 27, contained workers that were derived from multiple queens. In the following, we discuss five possibilities that can explain the presence of worker genotypes derived from multiple queens in *Lasius fuliginosus* colonies (see Gadau & al. 1998): (1) Intraspecific brood or worker raiding; (2) Primary polygyny as a result of pleometrosis; (3) Intranidal mating of related individuals; (4) Adoption of related queens after the mating flight; (5) Adoption of unrelated queens by orphaned colonies.

(1) By means of intraspecific brood or worker raiding, genotypes of foreign queens could be present in a monogynous colony. However, intraspecific raiding is not known for any *Lasius* species. Moreover, no other *Lasius fuliginosus* colonies, on which raids could have been conducted, were found in proximity to colony Lfu 1. Although colony Lfu 27 was located only about 30 meters apart from colony Lfu 28, no worker genotypes present in colony Lfu 28 were found in colony Lfu 27 (we assumed that colonies Lfu 27 and Lfu 28 were distinct colonies because no trail connection existed between them, they did not share any genotypes among workers, and alleles were present in both colonies that could not be found in the other one). Therefore, it is unlikely that intraspecific raids were conducted by *L. fuliginosus* in our study population.

(2) Pleometrosis can lead to the presence of several queen genotypes in a colony as a result of primary polygyny. However, in most species that exhibit pleometrosis only one queen remains in the colony after the emergence of the first workers (Bourke & Franks 1995). Hence, primary polygyny is rare in ants and has unlikely occurred in colonies Lfu 1 and Lfu 27. However, even if pleometrosis occurred but did not lead to primary polygyny in the colony, it would be possible that we saw genotypes of several queens because of it. This would be the case if we sampled workers right after the colony founding, and before or shortly after the killing of all but one queen took place. In this way, workers derived from multiple founding queens could still be present in the colony. A newly founded colony would show relatively few *Lasius fuliginosus* workers and could be recognized by the presence of host workers. Since the colonies Lfu 1 and Lfu 27 were large, exhibited extensive trails and did not show any host workers, they were not incipient but fully-grown colonies. In addition, it is not clear if pleometrosis occurs in *L. fuliginosus* at all. For example, we found *L. umbratus* (Nylander, 1846) workers in the nest of colony 18 foraging with *L. fuliginosus* workers, which might indicate that this colony was newly founded. However, the colony was clearly monogynous and monandrous. Therefore, pleometrosis does not seem to be the reason for genotypes of multiple queens in colonies Lfu 1 and Lfu 27. Nevertheless, we do not exclude that parasitic pleometrosis could facilitate colony founding in *L. fuliginosus* as indicated by Mattheis (2003). Regarding the high amount of monogynous colonies in the studied population, all colonies would have to reduce functional queen number to one after the founding period. Such queen reductions might include killing of queens by workers, fighting among queens or the renouncement of reproduction by some queens. The latter, however, is only exhibited in few ant species (Bourke & Franks 1995). To test for pleometrosis in *L. fuliginosus*, studies on the sociogenetic structure of more newly founded colonies will be necessary.

(3) Sexuals in ant species do not necessarily participate in nuptial flights to mate. For several formicine species intranidal mating is known (e.g., Rosengren 1983, van Loon & al. 1990, Sundström 1993). That is, sexuals do not leave the natal nest but mate with related individuals inside the nest. However, in *Lasius fuliginosus*, extensive nuptial flights were observed by Mattheis (2003). The prevalence of independent colony foundation and nuptial flights is also supported by the absence of isolation by distance among colonies in the study population. Intranidal mating often results in high rates of homozygosity due to inbreeding. We calculated homozygosity rates for all colonies (see Tab. S6). Colony Lfu 01 indeed showed elevated homozygosity rates compared to the average rates across monogynous colonies. However, this was not the case for Lfu 27. In summary, intranidal mating in *L. fuliginosus* seems unlikely but remains a possible explanation (especially in Lfu 01).

(4) Re-adoption of daughters is usually associated with changes in life history and mating behaviour. In species that show queen adoption, mating often occurs in, on or close to the nest so that the possibility of predation while relocating and re-entering the nest is minimized (Bourke & Franks 1995). *Lasius fuliginosus* sexuals participate in extensive nuptial flights during which they cover considerable distances. This would make returning to their natal nest difficult and costly. On the contrary, adoption of colony daughters in *L. fuliginosus* was reported by Donisthorpe (1915). However, it was not described by other authors and Donisthorpe (1915) did not give evidence for his claims. Although we cannot exclude daughter adoption to be present in *L. fuliginosus*, it seems unlikely in the studied population. Mitochondrial haplotype analyses might prove useful to shed light on the descent and relatedness of matrilines in polygynous colonies.

(5) Finally, we propose the adoption of unrelated queens as the most likely explanation for multiple queen genotypes in colonies Lfu 1 and Lfu 27. When the queen in a monogynous colony dies, the colony is usually no longer able to produce sexual offspring. However, the production of sexuals can continue either if workers start to reproduce or if new queens are adopted into the colony. Mattheis (2003) observed that *Lasius fuliginosus* workers show aggression towards foreign conspecific queens. After the queen's death in a monogynous colony these aggressions might decrease. Subsequently, there could be a period in which *L. fuliginosus* colonies would accept new queens to be adopted into the colony. Such a mechanism is described in *Solenopsis* and *Myrmica* (see Tschinkel & Howard 1978, Seppä 1994). Queen adoption would be in accordance with the longevity of *L. fuliginosus* colonies despite the small queen size (Donisthorpe 1915, Mattheis 2003). Moreover, genotyping of colony Lfu 6 revealed that new genotypes were present among workers in 2017 compared to 2016, suggestive of the presence of a new reproductively active queen in the colony. It is unlikely that this new queen was related to the old one because, based on worker genotypes, it showed alleles at the loci Lf 04 and Lf 05 that were not present in the old queen. Therefore, the adoption of an unrelated queen into colony Lfu 6 has likely occurred.

If adoption of unrelated queens evolved in *Lasius fuliginosus*, it should yield fitness advantages to both the adopted queen and adopting workers. Adoption is advantageous for adopted queens because *L. fuliginosus* colonies are large and have elaborate nests in tree cavities that are constructed with at least two mutualistic fungal species (Schlick-Steiner & al. 2008). By means of adoption, queens take advantage of this costly resource and avoid the critical stage of colony founding. However, it is worthy of note that an intruding gyne also faces complications such as worker hostility and a lowered expected fertility (Keller 1993). Workers would obtain an inclusive fitness advantage adopting a newly mated queen under queenless conditions if they were related to her. Even if this was not the case, adoption could result in a fitness benefit for workers. Gadau & al. (1998) proposed this for *Camponotus ligniperda* (Latreille, 1802), in which

sexual brood overwinters twice. If maturation time of sexuals is long as in *C. ligniperda*, sexual brood of an old but dead queen, which was related to the workforce, would still be present in the colony some time after the queen's death. This brood's survival rate and thus the inclusive fitness of workers could be increased if a newly adopted queen produced additional workers. However, there is no information on the maturation time of sexuals in *L. fuliginosus* and whether the queens in the studied colonies were related to one another and to the workers.

Unrelated *Lasius fuliginosus* queens could also enter a colony against its fitness interests as intraspecific parasites. Such a scenario is likely since *L. fuliginosus* is well adapted to a parasitic lifestyle because of its mode of colony founding (temporary social parasitism in the *Chthonolasius* group). It is possible that *L. fuliginosus* queens can enter a foreign but conspecific colony and kill the queen in this colony as they do in colonies of their host species. This would present intraspecific parasitic behaviour. Since Mattheis (2003) observed high worker aggression towards foreign queens under queenright conditions parasitizing a *L. fuliginosus* colony might only be possible in orphaned colonies because defence mechanisms would be weaker there.

Our genetic data suggest that at least two queens would have been adopted into colony Lfu 1 and one queen in colony Lfu 27 (alleles of a dead queen could still be present in worker genotypes). This raises the question whether queen adoption in colony Lfu 1 led to a stable coexistence of multiple queens as shown for *C. ligniperda* by Gadau & al. (1998) or whether eventually all but one queen would be killed. To answer this question, the polygynous colonies need to be genotyped over consecutive years.

Mating frequency: We showed that the majority of queens in the studied *Lasius fuliginosus* population was singly mated. Colonies Lfu 13 and Lfu 33 were the only colonies for which a doubly mated queen was probable. This resulted in a population-wide effective mating frequency of $m_e = 1.017$. Boomsma & Ratnieks (1996) proposed four categories to classify mating frequencies in ants: (s) single (double mating is absent or rare; $m_e < 1.05$); (s - d) single-double (double mating occurs in 20% - 50% of queens; $m_e = 1.05$ to 1.25); (s-m) single-multiple (mating frequency above two occurs regularly; $m_e = 1.4$ to 2); and (m) multiple (mating frequency greater than two; $m_e > 2$). Even though over half of the ant species investigated show multiple mating to some degree (Bourke & Franks 1995), most species belong to categories (s) or (s-m) including *Lasius flavus* (Fabricius, 1782), *L. neglectus* and *L. niger* (Linnaeus, 1758). Instances of obligate multiple mating have been shown in the genera *Acromyrmex* (see Boomsma & al. 1999), *Atta* (see Boomsma & Ratnieks 1996), *Cardiocondyla* (see Lenoir & al. 2006), *Cataglyphis* (see Pearcy & al. 2009), *Pogonomyrmex* (e.g., Wiernasz & al. 2004) and in army ants (e.g., Kronauer & al. 2004, 2006), but have not been reported for *Lasius*. Therefore, it is not surprising that *L. fuliginosus* queens are generally mated once (category (s)).

Worker reproduction: There is evidence for worker reproduction in more than 40 species across 23 genera including several species within the Formicinae (mostly *Formica*) (reviewed by Bourke 1988). However, in the genus *Lasius* only *L. niger* workers were shown to produce males. We found evidence for worker reproduction in three of twelve *L. fuliginosus* colonies, now presenting the second *Lasius* species for which worker reproduction is documented.

Queen presence / absence is key for considering fitness costs and benefits associated with worker reproduction. In queenright colonies there is potential conflict over male parentage between workers and queens, particularly in monogynous and monandrous systems where workers are more closely related to their sons ($r = 0.5$) and nephews ($r = 0.375$) than to their brothers ($r = 0.25$) and should therefore favour their own male offspring over the queen's male offspring. However, male production could also have indirect negative effects on worker fitness by decreasing colony-level productivity (Keller & Nonacs 1993). Hence, queens are likely selected to prevent worker reproduction. Whether this occurs against the fitness interests of workers (manipulation) or not (honest signalling) is controversial (Keller & Nonacs 1993, Brunner & al. 2011). For queenless colonies, however, worker reproduction is the only remaining possibility to increase the fitness of both, the absent queen and the workers (Alexander 1974). Therefore, reproductive conflicts are alleviated under queenless conditions, rendering worker reproduction potentially adaptive. Indeed, most cases of worker reproduction are reported under queenless conditions (Hölldobler & Wilson 1990). Because of the potential conflict over male parentage and the higher probability of queen orphanage in monogynous systems, Bourke (1988) suggested that worker reproduction might be more frequent in monogynous than in polygynous species. Since all colonies with worker-derived males in the studied population, i.e., colonies Lfu 6, Lfu 8 and Lfu 19, were monogynous (and monandrous), our findings support this hypothesis. It remains to be seen whether worker reproduction has occurred under queenless or queenright conditions in these colonies.

In colonies Lfu 6 and Lfu 19 it was likely that all sampled males were produced by workers. Under queenright conditions worker reproduction in ants is commonly suppressed behaviourally or through pheromones (Fletcher & Ross 1985). This means that even if worker reproduction took place in a queenright colony, the fraction of worker-derived males should be low. Therefore, we hypothesized that colonies Lfu 6 and Lfu 19 were orphaned. If this was the case, either worker numbers would decline, or a new queen would be adopted in the years after male sampling. We genotyped workers of colony Lfu 6 that were sampled one year after initial worker and male sampling to see whether queen adoption took place in this colony. Indeed, new worker genotypes were found at all loci, suggesting that worker reproduction is a possible temporary stage between queen orphanage and queen adoption. To finally see whether colonies Lfu 6 and Lfu 19 were orphaned at the time of male sampling, it will be necessary to monitor these colonies in the years to come.

Queen orphanage is no possible explanation for worker reproduction in colony Lfu 8 since it was likely that most male offspring was produced by a queen. Only one male out of 31 was clearly worker-derived. This would be expected under queenright conditions because of the suppression of worker reproductive behaviour by the queen. As done for colony Lfu 6, we genotyped workers of colony Lfu 8 sampled one year after initial sampling. We did not find any new alleles at the loci Lf 03 to Lf 06 suggesting that no queen adoption took place in this colony (as would be expected).

Considering that in colony Lfu 8 only one male was identified as worker-derived and that the sampling size of males in all the other colonies was only 65% of the sampling

size in colony Lfu 8, it might well be possible that worker reproduction might be a feature of a greater number of colonies but was not detected due to too small sample sizes in this study. We propose that worker reproduction in *Lasius fuliginosus* occurs under both queenright and queenless conditions. With a queen present, only a small fraction of workers successfully produces males. After colony orphanage worker reproduction can become more frequent because behaviour and / or pheromones of the queen that supress worker reproduction are no longer present.

Outlook: Surprisingly, for a species that has large and long-lived colonies, *Lasius fuliginosus* appears to be predominantly monogynous and monandrous. However, we did record some cases of polyandry and workers which were derived from multiple queens in the study population. Latter might be the result of queen adoption by orphaned colonies, but this hypothesis needs further investigation. Our data challenge the polygyny hypothesis in *L. fuliginosus* as stated in the literature. Studies in more *L. fuliginosus* populations will be necessary to unveil the predominant queen number of this species.

We also found evidence for worker reproduction in *Lasius fuliginosus*, making it the second species in the genus *Lasius* for which this phenomenon is described. Worker reproduction might rarely occur in queenright *L. fuliginosus* colonies but might be a strategy to increase colony fitness after orphanage, potentially before new queens would be adopted. In recent years, not much work has been conducted on worker reproduction in ants and not many species have been studied in this regard at all. However, it seems to be a frequent feature (Hölldobler & Wilson 1990), and its implications for evolutionary processes, like retention or loss of worker ovaries, are still not well understood.

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