

THE EVOLUTION OF MULTIPLE MATING IN ARMY ANTS

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The evolution of mating systems in eusocial Hymenoptera is constrained because females mate only during a brief period early in life, whereas inseminated queens and their stored sperm may live for decades. Considerable research effort during recent years has firmly established that obligate multiple mating has evolved only a few times: in *Apis* honeybees, *Vespula* wasps, *Pogonomyrmex* harvester ants, *Atta* and *Acromyrmex* leaf-cutting ants, the ant *Cataglyphis cursor*, and in at least some army ants. Here we provide estimates of queen-mating frequency for New World *Neivamyrmex* and Old World *Aenictus* species, which, compared to other army ants, have relatively small colonies and little size polymorphism among workers. To provide the first overall comparative analysis of the evolution of army ant mating systems, we combine these new results with previous estimates for African *Dorylus* and New World *Eciton* army ants, which have very large colonies and considerable worker polymorphism. We show that queens of *Neivamyrmex* and *Aenictus* mate with the same high numbers of males (usually ca. 10–20) as do queens of army ant species with very large colony sizes. We infer that multiple queen mating is ancestral in army ants and has evolved over 100 million years ago as part of the army ant adaptive syndrome. A comparison of army ants and honeybees suggests that mating systems in these two distantly related groups may have been convergently shaped by strikingly similar selective pressures.

KEY WORDS: Aenictinae, *Aenictus*, colony fission, diploid male load, Ecitoninae, inbreeding, *Neivamyrmex*, polyandry.

Elevated predation risk during courtship and copulation may impose significant costs on females when mating with multiple males (polyandry). These costs may be augmented when prolonged mating efforts exhaust irreplaceable energy resources, increase exposure to sexually transmitted diseases, or impose fitness costs because of sperm competition or metabolic costs of storing more sperm (e.g., Thornhill and Alcock 1983; Sherman et al. 1988; Simmons 2001). To be adaptive, the fitness benefits of multiple mating should outweigh these cumulative costs. Issues of mate choice are particularly acute in the eusocial insects (ants, some bees, some wasps, and termites) where sexual partners commit themselves for life (Boomsma et al. 2005a).

Obligate multiple mating of hymenopteran queens has been documented in leaf-cutting ants (genera *Atta* and *Acromyrmex*; Villesen et al. 2002), harvester ants (genus *Pogonomyrmex*; Rheindt et al. 2004; Wiernasz et al. 2004), honeybees (genus *Apis*;

Moritz et al. 1995; Palmer and Oldroyd 2000; Tarpay et al. 2004), vespine wasps (genus *Vespula*; Ross 1986; Foster and Ratnieks 2001), the ant *Cataglyphis cursor* (Percy et al. 2004), and some army ants (genera *Dorylus* and *Eciton*; Denny et al. 2004; Kronauer et al. 2004, 2006a,b). In the first four groups, polyandry has been studied in detail. It has a single evolutionary origin in each case and was subsequently maintained in the descendent lineages of each respective clade. Only one reversal to single mating is known, an inquiline social parasite of *Acromyrmex* leaf-cutting ants (Sumner et al. 2004).

The four most widely applicable hypotheses for the evolution of polyandry in eusocial Hymenoptera are: (1) worker-caste determination has a genetic component so that polyandrous species can develop more complex caste systems because they have genetically diverse colonies (Page et al. 1989), (2) genetically diverse colonies are better able to cope with parasites (Sherman

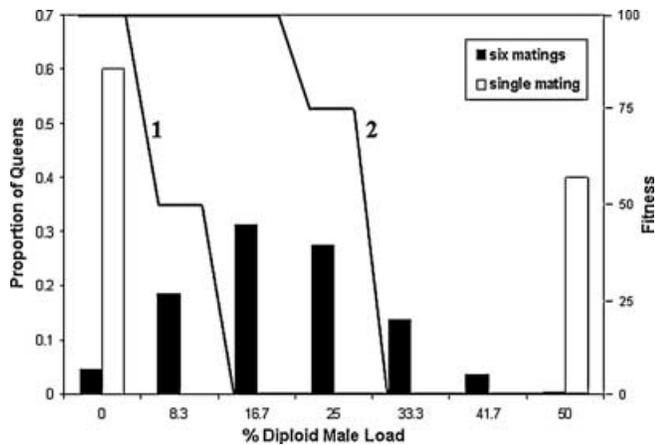


Figure 1. The distribution of diploid male load among queens with one (white bars) and six (black bars) matings assuming single-locus complementary sex-determination, no inbreeding, five equally common sex-locus alleles in the population, and equal contributions of all males to the offspring (calculated following Page 1980). Two hypothetical fitness functions for queens with different levels of diploid male load (DML) are given (labeled 1 and 2). Function 1 assumes that even low levels of DML are detrimental, whereas they have no effect on fitness under function 2. The fitness of queens mated to one and six males, respectively, was determined for each fitness function by multiplying the proportion of queens having a certain amount of DML with the respective fitness and summing over all possible values of DML. Under fitness function 1, single mating is favored over multiple mating by a factor of 4.3 (by a factor of 1.27 if we assume 20 sex-locus alleles; not shown), whereas multiple mating is favored over single mating under function 2 by a factor of 1.25 (1.11 with 20 sex-locus alleles; not shown). The average amount of DML is independent of the number of matings.

et al. 1988; Schmid-Hempel 1994, 1998; Boomsma et al. 2005b), (3) queens mate with multiple males to acquire more sperm (Cole 1983), and (4) polyandry can be adaptive to reduce fitness load from sterile diploid males (Page 1980; Fig. 1). Colony size has often been discussed as a correlate of polyandry (e.g., Cole 1983; Crozier and Page 1985; Boomsma and Ratnieks 1996), because larger colonies need queens with more stored sperm (Cole 1983) and workers that specialize on more diverse tasks and that can cope with larger numbers of parasites (Wilson 1975; Schmid-Hempel 1998; Boomsma et al. 2005b). These hypotheses, which are not mutually exclusive, have been amply reviewed, but a general synthesis has not been achieved in spite of the specific selection pressures and fitness benefits that characterize them (Bourke and Franks 1995; Boomsma and Ratnieks 1996; Crozier and Fjerdingstad 2001; Strassmann 2001; Brown and Schmid-Hempel 2003). Perhaps the only generalization that has become widely adopted is that Cole's (1983) sperm limitation hypothesis is now considered unlikely as a general explanation, because there is no good reason why males should not be able to evolve sufficient

sperm supplies to fully inseminate a queen (e.g., Crozier and Page 1985; but see Schlüns et al. 2005). This implies that comparative analyses of mating system transitions mapped on phylogenetic trees are essential to disentangle the significance of these alternative hypotheses for the evolutionary origins of multiple mating.

Recent empirical studies on leaf-cutting and harvester ants have mainly highlighted benefits of multiple queen mating that relate to social complexity via the expression of more complex polymorphism and polyethism in worker castes and an increased colony-level resistance to parasites (Hughes et al. 2003; Hughes and Boomsma 2004; Wiernasz et al. 2004). Also genetically diverse honeybee colonies have been shown to have more efficient worker polyethism and to be more resistant to parasites (Taryp 2003; Jones et al. 2004), but several authors favor the idea that these are secondary developments after multiple mating evolved initially in response to selection pressure from diploid male load (e.g., Crozier and Page 1985; Palmer and Oldroyd 2000).

Natural History of Army Ants

Obligate polyandry in army ants has been suspected for a long time (Raignier and Van Boven 1955; Rettenmeyer 1963), but has only recently been documented for representatives of two of the major clades, the African species *Dorylus (Anomma) molestus* (Kronauer et al. 2004, 2006a) and the Neotropical species *Eciton burchellii* (Denny et al. 2004; Kronauer et al. 2006b). Queens of both species have to mate with several males to become fully inseminated, but we have argued that this is probably a consequence of obligate polyandry, rather than the primary reason for its evolution (Kronauer and Boomsma 2007). Phylogenetic studies have further shown that army ants comprise a monophyletic group that originated just over 100 million years ago (Brady 2003; Moreau et al. 2006; Fig. 2). Army ants are keystone predators in tropical ecosystems and feed mainly on other arthropods, which they hunt and overwhelm in massive swarm raids. A likely evolutionary scenario is that the switch to obligate collective foraging and group predation implied that army ants had to become nomadic to avoid rapid depletion of local food resources. This probably made large colonies and reproduction by colony fission inevitable, because group predation is ineffective for small incipient colonies with independently founding queens. Colony fission in turn has been theoretically shown to select for highly male-biased numerical sex ratios (Macevicz 1979; Bulmer 1983). Army ant queens also became morphologically specialized: they lost their wings and specifically adapted to dependent colony founding and nomadism (Gotwald 1995; Brady 2003). Being the largest ants on Earth, African *Dorylus (Anomma)* queens also hold the world record in reproductive potential among the insects, with an egg-laying capacity of several millions per month (Raignier and Van Boven 1955; Hölldobler and Wilson 1990; Gotwald 1995). Army

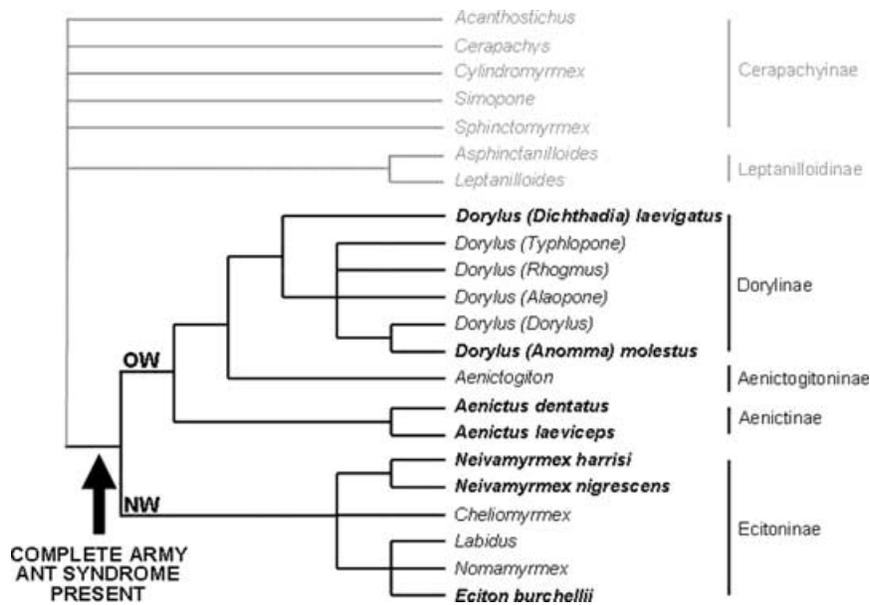


Figure 2. Phylogeny of the dorylomorph clade, which includes the army ants (in black) and their putative closest relatives (in gray), based on Brady (2003) and Brady and Ward (2005). The common ancestor of the monophyletic army ants has been inferred to have had all the traits that characterize the army ant adaptive syndrome that is shared by all extant species (Brady 2003). The separation between the New World (NW) and Old World (OW) clades dates back to an ancient split about 105–110 million years ago, after the army ant syndrome had evolved (Brady 2003; Moreau et al. 2006). Only the males are known for Aenictogitoninae, so that it remains unclear to what extent the workers exhibit army ant behavior. Monophyly of the dorylomorphs has been further supported by Ward (2005), but the genus *Cerapachys* may not be a clade (Brady and Ward 2005; Moreau et al. 2006). All army ant species for which information on queen-mating frequency is available from the present or previous studies are given in bold-face text (see also Table 4 and text).

ant queens never leave the protection of the colony, where they mate with foreign incoming males which disperse on the wing. This entire suite of interrelated life-history traits characterizes all extant army ants. It is referred to as the army ant adaptive syndrome and originated once in a common ancestor of all army ants (Brady 2003; Fig. 2).

The species of *Dorylus* (*Anomma*) and *Eciton* where multiple queen mating has been documented (Kronauer 2004, 2006a, 2006b; Denny et al. 2004) have extreme degrees of worker caste polymorphism and larger colonies than almost any other social insect: around 15 million workers in *Dorylus* (*Anomma*) and close to 1 million in *E. burchellii* (Schneirla 1971). In the present study we provide detailed estimates of queen-mating frequency in North American *Neivamyrmex* and Southeast Asian *Aenictus* army ants, which exhibit little or no worker polymorphism, respectively, and have relatively small colonies (with a colony size of ca. 100,000 workers (Schneirla 1971) the two species have small colonies compared to the other army ant species mentioned, but their colonies are still very large compared to other eusocial Hymenoptera in general). These two genera also belong to clades of the army ant phylogeny (Fig. 2) that are very informative for a comparative analysis of army ant mating system evolution.

OBJECTIVES OF THE PRESENT STUDY

Our new data on the mating systems of two army ant genera that have not been investigated before allow us to infer whether

polyandry has evolved once, as is the case in the other groups of polyandrous eusocial Hymenoptera, or several times independently, and to assess where on the phylogeny mating-system transitions have taken place. These basic data further allow us to identify independent phylogenetic contrasts to compare mating frequencies between army ants that differ in colony size and worker polymorphism. We evaluate the general significance of these insights by comparing army ants with *Apis* honeybees and *Melipona* stingless bees (with typical colony sizes of several thousands to tens of thousands, and several hundreds to thousands, respectively), which also reproduce by colony fission and have highly male-biased numerical sex ratios. Finally, because inbreeding has been hypothesized to augment selection for polyandry under hypotheses 1 and 2 (Bourke and Franks 1995) and to increase the likelihood of matched matings under hypothesis 4 (see above), we provide the first detailed analysis of inbreeding in army ants.

Material and Methods

SAMPLED MATERIAL

We analyzed queen-mating frequencies for two species of *Neivamyrmex* (subfamily Ecitoninae) and two species of *Aenictus* (subfamily Aenictinae). Samples of workers were collected from ten colonies of *N. nigrescens* Cresson in Arizona (United States) and Sonora (Mexico) and from one colony of *N. harrisi*

Table 1. Collection localities and reference collection numbers for the *Neivamyrmex nigrescens* (Nni), *N. harrisi* (Nha), *Aenictus laeviceps* (Ala), and *A. dentatus* (Ade) samples used in this study. Reference material has been deposited in the Robert A. Johnson collection, Tempe, Arizona, and at the Zoological Museum in Copenhagen. Arizona is abbreviated AZ.

Colony	Locality	Latitude	Longitude	Elevation (m)	Collection date	Collection no.
Nni1	USA, AZ, Santa Cruz	31°23'N	111°03'W	1230	6/4/1998	RAJ1329
Nni2	USA, AZ, Maricopa	33°21'N	111°58'W	410	17/8/1998	SWR1544
Nni3	USA, AZ, Santa Cruz	31°26'N	110°39'W	1580	10/4/1999	RAJ1805
Nni4	USA, AZ, Gila	33°33'N	110°42'W	1480	4/5/1999	RAJ1823
Nni5	USA, AZ, Cochise	31° 55'N	109°12'W	1670	9/5/2004	RAJ3424
Nni6	Mexico, Sonora, Mazatán	29°05'N	110°10'W	1550	28/4/2004	RAJ3412
Nni7	USA, AZ, Apache	34°01'N	109°11'W	2270	11/10/2004	RAJ3487
Nni8	USA, AZ, Santa Cruz	31°27'N	110°38'W	1530	30/10/2004	RAJ3516
Nni9	USA, AZ, Cochise	31°55'N	109°14'W	1650	8/2002	CS02/17
Nni10	USA, AZ, Cochise	31°55'N	109°14'W	1650	15/8/2002	CS02/34
Nha1	USA, AZ, Maricopa	33°43'N	111°24'W	1260	3/8/2002	DK39
Ala1	Malaysia, Sabah, Tawau Hills Park	4° 28'N	117° 55'E	–	30/10/2002	Ala1
Ala2	Malaysia, Sabah, Poring Hot Springs	5° 59'N	116° 41'E	550	24/9/2002	Ala2
Ala3	Malaysia, Sabah, Poring Hot Springs	5° 59'N	116° 41'E	550	19/8/2002	Ala3
Ala4	Malaysia, Sabah, Tawau Hills Park	4° 28'N	117° 55'E	–	4/10/2002	Ala4
Ala5	Malaysia, Sabah, Poring Hot Springs	5° 59'N	116° 41'E	550	16/7/2004	Ala5
Ade1	Malaysia, Sabah, Tawau Hills Park	4° 28'N	117° 55'E	–	20/10/2002	Ade1

Haldeman in Arizona between April 1998 and October 2004 (Table 1). *Aenictus* workers were collected from five colonies of *A. laeviceps* Smith and one colony of *A. dentatus* Forel in Malaysia (Table 1). The two species of *Aenictus* belong to distinct clades within the Aenictinae (Wilson 1964). Ants were stored in 96% ethanol prior to analyses.

LABORATORY PROTOCOLS

DNA was extracted following a standard protocol using Chelex[®] 100 (BioRad, Hercules, CA) resin. We first genotyped 541 workers from the 10 colonies of *N. nigrescens* for three microsatellite loci (*NeiA*, *NeiB*, *NeiC*) that were multiplexed for PCR amplification and size determination of products on an ABI377 automatic sequencer (Applied Biosystems, Foster City, CA). A total of 280 individuals were also genotyped for a fourth locus (*NeiD*) to increase detection power for patriline (groups of full sister workers) in two colonies, and to improve the accuracy of regression relatedness estimates in the whole dataset. This sample included all individuals from colonies Nni7 (which had the lowest number of patrilines) and Nni6 (the only colony that contained workers from two matriline, i.e., daughters of two queens), and at least one individual per patriline from all remaining colonies. All four microsatellite loci were highly polymorphic in *N. nigrescens* with seven to 30 (mean 18.8) alleles and expected heterozygosities from 0.54 to 0.95 (mean 0.8). We genotyped 21 workers from one colony of *N. harrisi* for loci *NeiA* (monomorphic), *NeiB* (three alleles), and *NeiC* (four alleles). Locus *NeiD* did not amplify in this species.

We used the microsatellite loci *AenA*, *AenB*, *AenC*, *AenD*, and *AenE* to genotype 229 workers from five colonies of *A. laeviceps* and locus *AenF* to genotype a subset of 104 workers that were evenly distributed among the colonies. This subset was genotyped for locus *AenF* mainly to characterize the allelic diversity at that locus. These six loci had two to 17 alleles (mean 8.0) and expected heterozygosities from 0.48 to 0.89 (mean 0.66). Thirty-five workers from one colony of *A. dentatus* were genotyped for loci *AenG* and *AenH* with eight and 13 alleles in that colony, respectively. Genotyping protocols and marker properties will be detailed elsewhere (Kronauer et al., in press).

DATA ANALYSIS

We first checked whether all workers from a given colony could be the offspring of a single queen. In one case where one queen could not account for all worker genotypes, we manually deduced the genotypes of a mother and a daughter queen that together could have produced the workers, following the procedure outlined in Kronauer et al. (2004). Worker genotypes within single matriline of social insects are interdependent due to common ancestry, which can confound population genetic analyses if workers are treated as independent samples. Consequently, we estimated allele frequencies using a derived dataset that contained only the deduced queen and male genotypes. First, we inferred genotypes of queens and their mates using the computer program MATESOFT version 1.0 (Moilanen et al. 2004) while assuming equal frequencies for each allele at a given locus. The MATESOFT output was inspected to detect potential scoring errors and we repeated the

entire genotyping procedure for all questionable individuals. In cases where different potential queen genotypes could account for the data, only the most likely queen and her inferred mates were entered into the derived dataset. For further analyses, queen genotypes were duplicated and haploid male genotypes were entered as diploids. This allowed us to include inferred males that could have had two possible alleles at a given locus as heterozygotes. Unbiased allele frequencies and expected heterozygosities were subsequently estimated with the computer program FSTAT version 2.9.3.2 (<http://www2.unil.ch/popgen/softwares/fstat.htm>; Goudet 1995). Observed heterozygosities were calculated directly from the worker genotypes because these are representative for diploid individuals in the population.

The unbiased estimates of background allele frequencies were used when reconstructing queen and male genotypes with their associated weighted probabilities in MATESOFT (see Kronauer et al. 2004). The same program was used to estimate the sum of squared paternity contributions following Pamilo (1993) and Pedersen and Boomsma (1999). The effective number of matings per queen, m_e (the number of equally contributing males that would have produced the same average relatedness among worker offspring), was estimated with algorithms by Starr (1984), Pamilo (1993), and Nielsen et al. (2003) and these estimates are referred to as m_{e1} , m_{e2} , and m_{e3} , respectively. Based on the worker genotypes, the background allele frequencies, and the segregation pattern of putative queen alleles among the workers, this two-step procedure allowed us to reconstruct both the most likely queen

genotype and any less likely alternative for each colony or matriline. In colonies where less likely alternatives could be inferred, these were taken into account by averaging mating-frequency statistics over all alternative queen genotypes weighted according to their respective probabilities.

Genetic regression relatedness (r) between queens and their mates (r_{qm} ; bidirectional averages), between the mates of a single queen (r_{mm}), and between workers of single matrilines (r_{ww}) were estimated by the program Relatedness version 5.0.8 (<http://www.gsoftnet.us/GSoft.html>; Queller and Goodnight 1989). Only the most likely queen and male genotypes were used for these calculations. Estimates of r_{ww} were then compared to the expected values based on Starr (1984; m_{e1} in the present study), which were calculated according to Pamilo (1993):

$$r_{ww} = 0.25 + 0.5/m_{e1}.$$

The estimated worker-worker regression relatedness (r_{ww}) should be identical to the relatedness estimate based on pedigree information (m_{e1}) if the assumption of zero relatedness between male and female parents (i.e., no inbreeding) is met. The two estimates were therefore compared using paired t -tests. All relatedness estimates are given with standard errors obtained from jackknifing over colonies. We used 2-tailed t -tests to check relatedness coefficients for significant deviations from zero. Regression relatedness analyses were not performed in *Aenictus* due to the smaller sample size and the disjunct distribution of samples.

Table 2. Observed and effective mating frequencies of 11 *Neivamyrmex nigrescens* queens: n : number of worker offspring analysed; k_{obs} : observed number of patriline; m_e : effective number of patriline estimated from the sum of squared paternity contributions following Starr (1984; m_{e1}) and using the sample size corrections given in Pamilo (1993; m_{e2}) and Nielsen et al. (2003; m_{e3}). In cases where alternative queens could explain the worker genotypes, mating frequencies are given as averages over all possible queens weighted by their alternative probabilities determined in MATESOFT. Average worker-worker relatedness (r_{ww}) was calculated following Queller and Goodnight (1989). Letters (a, b) denote the two related queens that were inferred for colony Nni6 and their assigned offspring. Overall values are arithmetic means \pm SE for k_{obs} and harmonic means \pm SE for m_e , with standard errors obtained by jackknifing over queens.

Queen	n	k_{obs}	m_{e1}	m_{e2}	m_{e3}	r_{ww}
Nni1	51	16	13.1	17.2	17.0	0.43
Nni2	51	17	13.6	18.3	18.0	0.35
Nni3	46	15	11.0	14.2	14.0	0.33
Nni4	50	13	8.0	9.3	9.2	0.32
Nni5	50	13	8.7	10.3	10.2	0.26
Nni6a	39	14	8.8	11.1	10.9	0.30
Nni6b	61	22	16.4	22.0	21.8	0.33
Nni7	50	11.9	7.3	8.4	8.4	0.55
Nni8	50	17	13.0	17.2	17.0	0.35
Nni9	71	13	11.3	13.2	13.2	0.26
Nni10	22	12	8.6	13.6	12.9	0.23
Overall	541	14.9 \pm 0.9	10.2 \pm 0.8	12.9 \pm 1.2	12.8 \pm 1.1	0.35 \pm 0.03

Table 3. Observed and effective mating frequencies of five *Aenictus laeviceps* queens (see the heading of Table 2 for further details).

Queen	<i>n</i>	<i>k</i> _{obs}	<i>m</i> _{e1}	<i>m</i> _{e2}	<i>m</i> _{e3}
Ala1	50	17.0	12.3	16.0	15.8
Ala2	50	18.0	13.6	18.3	18.0
Ala3	45	17.9	15.0	22.0	21.5
Ala4	34	15.0	12.3	18.8	18.2
Ala5	50	21.0	15.5	21.9	21.6
Overall	229	17.8±1.0	13.6±0.7	19.1±1.2	18.8±1.1

Results

NUMBER OF QUEENS CONTRIBUTING TO THE WORKER OFFSPRING

Contributions to the workers from both a mother and a daughter queen are expected in army ant colonies for a short time after colony fission (Kronauer et al. 2004). Worker genotypes from all but one colony could be explained by a single queen, indicating that no recent queen displacement had taken place. The exception was colony Nni6 where 100 of the 102 workers could together be accounted for by a mother and daughter queen; the remaining two workers required the assumption of an additional unrelated queen. Genotyping of these two workers was repeated with identical results, ruling out genotyping errors. As both genotypes were inconsistent with the two matrilines at multiple loci, they could not be explained by single mutations and were thus excluded from further analyses as probable drifters between colonies.

QUEEN-MATING FREQUENCY

The number of males that contributed to a queen's offspring was consistently high. Observed (*k*_{obs}) and effective (*m*_{e3}) mating frequencies in *N. nigrescens* ranged from 11.9 to 22 (arithmetic mean ± SE: 14.9 ± 0.9) and 8.4 to 21.8 (harmonic mean ± SE: 12.8 ± 1.1), respectively. Alternative measures (Starr 1984; Pamilo 1993) gave similar estimates (Table 2). Genotyping workers for the fourth locus (*NeiD*) did not identify any additional patriline for queens Nni6a and Nni6b, but added one patriline for queen Nni7. Adding this one patriline to the 47 patrilines already identified for the three queens implied that adding the fourth locus increased the number of detected mates by just 2%. We therefore conclude that our non-detection error due to limited allelic variation at marker loci was very low even when using only three microsatellite markers. Assuming that the mates of each queen were unrelated, this non-detection error (*P_r*) can be estimated as the product of the summed squared allele frequencies for each locus (Boomsma and Ratnieks 1996), which gave *P_r* = 0.005 for three loci and *P_r* = 0.0004 when including locus *NeiD*.

All workers in the single colony of *N. harrisi* could be accounted for by one multiply mated queen with *k*_{obs} = 4.1, *m*_{e3} = 3.9, and *m*_{e2} = 4.0. However, the non-detection error due to limited genetic variation was probably higher in this species, so that these figures are almost certainly underestimates.

Queens of *A. laeviceps* were also highly multiply mated with estimates for *k*_{obs} and *m*_{e3} ranging from 15 to 21 (arithmetic mean ± SE: 17.8 ± 1.0) and 15.8 to 21.6 (harmonic mean ± SE: 18.8 ± 1.1), respectively (Table 3). The overall, non-detection

Table 4. Overview of important life-history characteristics of the army ant species for which information on queen-mating frequencies is available. Colony size is given as the approximated "average" number of workers in normal mature colonies following Schneirla (1971) or based on a count from a single colony of *Dorylus laevigatus* (Berghoff et al. 2002). Other estimates for some of the species are available in the literature, but are not considered here because they give similar values. Queen size is body length (mm) in the contracted (non-physogastric) state. Worker polymorphism is given as the body size ratio of largest to smallest workers. Effective mating frequencies (Nielsen et al. 2003) are given as harmonic means with standard errors from jackknifing over colonies. Life-history data for *Dorylus (Anomma) spec.* refer to *D. wilverthi* whereas the mating frequency refers to *D. molestus*. The two species are closely related and are very similar in appearance and life history.

Species	Subfamily	Colony size (×10 ⁶)	Queen size	Worker polymorphism	Effective mating frequency (mean ± SE)	Reference for life-history data	Reference for mating frequency data
<i>Eciton burchellii</i>	Ecitoninae	0.9	21.0	3.1	12.9±1.1	Schneirla 1971	Kronauer et al. 2006b
<i>Neivamyrmex nigrescens</i>	Ecitoninae	0.12	12.5	1.8	12.8±1.1	Schneirla 1971	This study
<i>Dorylus (Anomma) spec.</i>	Dorylinae	15	52.0	3.7	15.9±2.8	Schneirla 1971	Kronauer et al. 2004, 2006a
<i>Dorylus (Dichthadia) laevigatus</i> ¹	Dorylinae	0.325	28.0	4.1	High	Berghoff et al. 2002	Winter 2004
<i>Aenictus laeviceps</i>	Aenictinae	0.1	8.1	1.2	18.8±1.1	Schneirla 1971	This study

¹No precise estimates of effective mating frequencies have been published for this species, but it is clear that they are similarly high. Winter (2004) reports an average *k*_{obs} of 9.5 over four colonies (total of 77 workers screened).

error for this dataset was $P_r = 0.0011$ for five loci and $P_r = 0.0006$ after including the additional locus *AenF*. For the single colony of *A. dentatus* we detected 19 mates and estimated an effective mating frequency of $m_{e3} = 25.9$ ($m_{e2} = 27.0$).

The data available (Table 4) give us two independent phylogenetic contrasts (Fig. 2) for comparing quantitative aspects of army ant mating systems. The first comparison concerns the New World species *Eciton burchellii* and *Neivamyrmex nigrescens* and the second the two Old World species *Dorylus molestus* and *Aenictus laeviceps*. They show that the mating frequencies of *E. burchellii* and *N. nigrescens* are essentially identical and that the estimates for *A. laeviceps* are equal to or even higher than those for *D. molestus* (Table 4).

INBREEDING AND RELATEDNESS OF COLONY MEMBERS IN *N. NIGRESCENS*

The average relatedness between colony workers was 0.35 ± 0.03 (Table 2) and did not differ from the expected value based on pedigree relatedness (0.30 ± 0.004 ; $P = 0.19$). The estimated expected heterozygosity across all colonies and loci was slightly higher than the observed value (0.799 vs. 0.759), resulting in a small positive inbreeding coefficient that was marginally significant ($F_{IS} = 0.050 \pm 0.023$ SE, $n = 11$ colonies; 2-sided t -test, $t = 2.23$, $P = 0.05$; 95% CI from bootstrapping over colonies: 0.020–0.092). As our samples were collected over a large and disjunct area (Table 1), this slight deficiency of heterozygotes is likely due to unconsidered population sub-structuring (the Wahlund effect; Wahlund 1928) rather than inbreeding by sib mating. The mates of each queen were on average neither related to each other ($r_{mm} = 0.02 \pm 0.02$; $P = 0.31$) nor to the queen that they had mated with ($r_{qm} = 0.03 \pm 0.02$; $P = 0.16$), reinforcing the conclusion that inbreeding is not a significant factor in explaining the genetic colony composition of *N. nigrescens*.

The genetic markers used were accurate enough to reconstruct directly also whether the daughter queen of colony Nni6 had mated with any of her brothers. The most likely interpretation was that the daughter queen had mated with 15 unrelated males (details are given in the Supplementary Material available online).

Discussion

THE ORIGIN AND EVOLUTION OF MULTIPLE MATING IN ARMY ANTS

Our finding that all sampled queens of *N. nigrescens*, *N. harrisi*, *A. laeviceps*, and *A. dentatus* mated with many males is consistent with results for *D. molestus* (Kronauer et al. 2004, 2006a), *D. laevigatus* (Winter 2004), and *E. burchellii* (Denny et al. 2004; Kronauer et al. 2006b). These results imply that (1) army ants almost certainly represent the largest known adaptive radiation of an obligatory polyandrous clade in the social insects, (2) a single early transition to multiple queen mating has taken place at

or before the base of the entire army ant clade, so that multiple queen mating is an integral part of the army ant adaptive syndrome (Fig. 2), and (3) army ant species with the largest colonies and the most extreme worker polymorphism are not characterized by higher queen-mating frequencies. This suggests that the level of multiple mating has not been further elaborated in connection to worker caste polymorphism or colony size after the initial evolutionary transition to polyandry.

Being part of the army ant adaptive syndrome, polyandry probably originated in close association with two other defining traits of the army ant reproductive syndrome that became established before further radiation took place: reproduction by colony fission and highly male-biased numerical sex ratios. These two interrelated traits are rare among eusocial Hymenoptera, but do occur in two other well-studied groups, the highly polyandrous honeybees and the monandrous stingless bees. In the following section we will compare these three independently evolved mating systems to evaluate whether they can be understood within a single framework of selective forces and constraints.

MULTIPLE MATING IN ARMY ANTS AND EUSOCIAL BEES

Honeybees are by far the best studied eusocial Hymenoptera, but their reproduction by colony fission, highly male-biased numerical sex-ratios, and very high queen-mating frequencies (Moritz et al. 1995; Palmer and Oldroyd 2000; Tarpay et al. 2004) have also been considered as odd exceptions, until they recently found their match in army ants (Denny et al. 2004; Kronauer et al. 2004, 2006a,b). In the honeybee, *Apis mellifera*, the evolution of polyandry can be explained as a response to the production of diploid males, which are sterile and impose a colony fitness cost because eggs and rearing efforts are wasted on individuals that can neither work nor reproduce (Fig. 1; Page 1980; Crozier and Page 1985; Ratnieks 1990; Pamilo et al. 1994; Palmer and Oldroyd 2000; Crozier and Fjerdingstad 2001; Tarpay and Page 2001, 2002). In addition to these well-known costs, we could add that diploid male production also imposes long term costs by wasting sperm, because queens do not re-mate later in life and are generally believed to be sperm-limited in their total lifetime fitness, at least in species that have large and long-lived colonies (Tschinkel 1987; Tschinkel and Porter 1988; Baer et al. 2006; Kronauer and Boomsma 2007).

Diploid males are produced if a queen mates with a male that carries one of her two complementary sex-determining (CSD) alleles (Beye et al. 2003). In most species with independent colony founding (e.g., the fire ant *Solenopsis invicta*), incipient colonies with any amount of diploid male load (DML) are likely to be outcompeted by neighboring colonies headed by queens without DML (Ross and Fletcher 1986; Ross et al. 1988; Crozier and Pamilo 1996). Monandry is therefore adaptive, because it

maximizes the proportion of young queens with zero DML (fitness curve 1 in Fig. 1). However, in honeybees and army ants that reproduce by colony fission, very few new queens are produced and they are richly endowed with a large fraction of colony workers that accompany them to form independent daughter colonies before their DML can be assessed. For colonies of these species, it is therefore important that low levels of DML can be tolerated, whereas high levels are likely to remain lethal as has been recently shown for honeybees (Tarpy and Page 2001, 2002). Polyandry is therefore adaptive, because it makes the proportion of young queens with high levels of DML negligible (fitness curve 2 in Fig. 1), thereby assuring that young colonies are unlikely to die from DML.

At present there is no direct evidence for DML in army ants, but this could be obtained (albeit indirectly) by comparing patriline frequencies among eggs and pupae to see whether the frequency of some patrilines is reduced by 50% during larval development. To assume that army ants suffer from DML, however, seems reasonable. The CSD system appears to be ancestral in aculeate Hymenoptera (ants, bees, and wasps) and has, as far as we know, not been lost in any eusocial species (Cook and Crozier 1995; Crozier and Pamilo 1996; Van Wilgenburg et al. 2006).

Melipona stingless bees, which are similar to army ants and honeybees in that they reproduce by colony fission and have highly male-biased numerical sex ratios, normally have single queen mating (Tóth et al. 2002). Although this seems to contradict the DML hypothesis outlined above, there is also an essential constraint in stingless bees that does not apply in honeybees or army ants. This is that brood cells are mass provisioned and sealed directly after egg laying, so that diploid male brood cannot be detected and removed at low cost early in development as has been documented for the honeybees *Apis mellifera* and *A. cerana* (Woyke 1963; Camargo 1982; Page and Kerr 1990; Ratnieks 1990). This suggests that the fitness penalties of diploid male production in *Melipona* spp. will resemble those of independently founding species (fitness curve 1 in Fig. 1). Because of this, *Melipona* queens have remained monandrous and workers have been selected to recognize and kill queens that produce diploid males and to rear a sister as replacement (Camargo 1979; Kerr 1987; Page and Kerr 1990; Ratnieks 1990). This combination of traits maximizes the proportion of young queens with zero DML, while avoiding the loss of highly valuable young colonies because of DML.

Another noteworthy case is the monogynous ant species *Cataglyphis cursor*, which also reproduces by colony fission and for which high and obligate levels of polyandry have recently been documented (Pearcy et al. 2004). Although this species tends to produce new queens parthenogenetically, the workers are sexually produced so that this derived social system may have responded to DML in similar ways as those of honeybees and army ants sup-

posedly have. Also several ponerine ants and the wasp *Provespa anomala* are known to reproduce by colony fission (Peeters and Ito 2001). The mating systems of these social insects have not been investigated, but will allow further tests of the possible connection between colony fission, DML, and the origin of multiple queen mating.

IS THE PRECISE HIGH NUMBER OF MATINGS ADAPTIVE?

Queens of both honeybees (Moritz et al. 1995; Palmer and Oldroyd 2000; Tarpy et al. 2004) and army ants (Kronauer et al. 2004, 2006a, 2006b; this study) often mate with many males, even though > 90% of the reduction in DML variance and > 80% of the maximal reduction in worker relatedness is achieved with only six matings (e.g., Palmer and Oldroyd 2000). To understand these high mating frequencies, it is important to realize that relative to species with independent colony founding, the costs of mating are probably low in all fissioning species, as numerical sex ratios are highly male-biased and queens are unlikely to be constrained in their mating opportunities by male availability. For honeybees it has been argued that once a queen has assured her ca. six inseminations, additional matings may not be adaptive, because queens cannot precisely count the number of inseminations and copulations happen in quick succession at low costs for queens (Ratnieks 1990; Tarpy and Page 2000, 2001). The cost of mating is likely to be even lower in army ants, where queens mate inside the colony while being protected by the workers (Kronauer et al. 2004). Kraus et al. (2005) further showed that queen-mating frequency in honeybees has a rather high additive genetic variance, so that the precise (high) level of polyandry can respond to selection. As life-history traits that are closely linked to fitness normally have little additive genetic variance, this finding is consistent with the very high queen-mating frequencies being selectively almost neutral (although Kraus et al. (2005) argue for balancing selection rather than selective neutrality to explain their results). We thus hypothesize that the exact high number of mates is also selectively neutral in army ants, and that the precise number of queen inseminations is determined by proximate factors such as the actual number of males entering the colony during the receptive phase of the queen and the very long duration of copulations (Kronauer and Boomsma 2007).

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