

Testing the genetic determination of the soldier caste in the silver ant

L. Leniaud¹ · M. Pearcy¹ · A. Taheri² · S. Aron¹

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Abstract Division of labor among workers is a hallmark of social insects that has largely contributed to their ecological success. In a number of species, ants in particular, environmental cues have long been recognized to determine the different phenotypes of workers. Recently, however, a genetic basis for worker polymorphism has been documented in some species. The silver ant *Cataglyphis bombycina* is characterized by the co-existence of two physiologically distinct castes of non-reproductive individuals: workers and soldiers. Soldiers are not a worker subcaste; they belong to a third caste, along with the queen and the worker castes. Using microsatellite DNA markers, we tested whether soldier caste determination has a genetic component, by comparing the distribution of patriline between the soldier and the worker castes. Our data show evidence of genotypic variation in caste propensity in only 2 out of 7 colonies sampled. In addition, most patrilines produce both workers and soldiers across all colonies. These results support moderate genotypic influence to soldier caste determination and suggest that non-genetic, likely environmental factors, also influence caste fate among non-reproductive offspring in this species. We also provide new estimates of the queen mating frequency, which support

biogeographic variations in mating behavior in *C. bombycina*.

Keywords Division of labor · Caste · Social insects · Mating frequency · *Cataglyphis*

Introduction

Division of labor amongst individuals has long been recognized as a key component of the tremendous ecological success of social insect societies (Bourke and Franks 1995; Hölldobler and Wilson 1990; Oster and Wilson 1978; Wilson 1971). It is best exemplified by the partitioning of reproduction between male and female sexuals, and sterile workers that carry out maintenance activities of the society such as sib-care, foraging, and colony defense. The division of labor is often associated with dramatic morphological and physiological differences between the reproductive and the worker castes. In some species, division of labor goes beyond reproduction and involves subcastes of workers that are characterized by distinct size, morphologies and behaviors (Fjerdingstad and Crozier 2006; Hölldobler and Wilson 1990; Oster and Wilson 1978). The occurrence of physical worker castes is relatively rare in social insects, but it has been described in about 15 % of ant genera (46/297; Hölldobler and Wilson 1990). In species with worker subcastes, task allocation is frequently accompanied by morphological adaptations: *major* workers with their large head and mandibles generally carry out tasks such as nest defense, seed milling or foraging, while *minor* workers tend to carry out tasks within the nest (Franks 1985; Hölldobler and Wilson 1990; Retana and Cerda 1994; Wetterer 1999).

Differentiation of workers into distinct morphs in ants has long been considered to stem from environmental

L. Leniaud and M. Pearcy contributed equally to this work.

✉ S. Aron
saron@ulb.ac.be
A. Taheri
amd.taheri@gmail.com

¹ Evolutionary Biology and Ecology, Université Libre de Bruxelles, Brussels, Belgium

² Ecology, Biodiversity and Environment, Abdelmalek Essaâdi University, Tétouan, Morocco

effects influencing developmental programs (reviewed in Molet et al. 2012; Nijhout and Wheeler 1982; Wheeler 1986; 1991). In line with this, worker size is thought to be determined by differential feeding, the effects of which are mediated by hormones to induce differential gene expression (Evans and Wheeler 2001; Wheeler 1991). However, cases of genotypic effects on worker caste fate have recently been uncovered in a number of ant species (reviewed in Anderson et al. 2008; Fjerdingstad and Crozier 2006; Schwander et al. 2010; Smith et al. 2008b). For example, workers produced by different patrines were shown to differ in their propensity to develop into *minor* or *major* workers in the leaf-cutting ants *Acromyrmex echinator* (Hughes and Boomsma 2007; Hughes et al. 2003) and *Atta colombica* (Evison and Hughes 2011; Holman et al. 2011), the seed harvester ant *Pogonomyrmex badius* (Rheindt et al. 2005; Smith et al. 2008a), the army ant *Eciton burchellii* (Jaffé et al. 2007), and *Pheidole rhea* (Huang et al. 2013). In these species, genetic effects on adult size (small or large) appear likely to interact with environmental cues to influence the propensity of larvae to develop into different worker subcastes (see e.g., Smith et al. 2008 for a detailed analysis on how genotypic and environmental factors combine to affect individual size and caste in the Florida harvester ant *P. badius*).

A remarkable feature of *Cataglyphis bombycina*—the “silver ant” of the Sahara, the Sinai and the deserts of the Arabian Peninsula—is the existence of two physiologically distinct castes of non-reproductive individuals within colonies: a polymorphic caste of workers that vary in size, and a monomorphic caste of soldiers (Délye 1957; Molet et al. 2014). Comparative studies of growth rules recently showed that soldiers are not a *major* worker subcaste (Molet et al. 2014). They belong to a third caste, the soldier caste, along with the queen and the worker castes. Soldiers are always bigger than workers and are characterized by large heads with highly developed scythe bladed mandibles (Fig. 1). Mean head size of soldiers is about 200 % larger than workers'. In contrast with workers, soldiers do not take part in outside activities; rather, they actively defend the colony when disturbed by firmly biting intruders. Their long palps allowing the transport of big sand pellets suggests that they could also be involved in nest construction (Bernard 1951).

C. bombycina colonies are headed by a single queen that is mated with several males (Leniaud et al. 2013). Previous microsatellite genotyping analyses suggested a genetic contribution to worker or soldier caste determination (Leniaud et al. 2013). However, this result called for some caution because it concerned a single colony with a small sample of individuals. Here, we tested the hypothesis of a genotypic component to soldier caste determination, by comparing the distribution of patrines between the soldier



Fig. 1 Worker and soldier of the silver ant *Cataglyphis bombycina*. Colonies contain a polymorphic caste of workers that vary in size, and a monomorphic caste of large soldiers with large heads and saber-shaped mandibles. Photo © Fernando Amor

and the worker castes in a larger sample of colonies. We also provide revised estimates of queen mating frequency in *C. bombycina*.

Materials and methods

The silver ant is common in the sand dunes of North Africa. Colonies are large, with a widespread network of galleries, and queens and soldiers are usually located in the deeper parts of the nest. Samples from seven colonies were collected in sand dunes at Merzouga (Morocco). Dunes are public land and no formal authorization was required to collect the ants; moreover, the silver ant is not a protected or endangered species. In mature colonies, soldiers represent only 1–2 % of the work force (S.A., pers. obs.), strongly limiting the number of soldiers sampled. Workers and soldiers excavated from each colony were immediately stored in 99.8 % ethanol for subsequent genetic analyses.

The genotype of workers and soldiers was determined at seven microsatellite loci previously developed for the species *C. cursor* (*Ccur99*, *Ccur93*, *Ccur11*, *Ccur89*; Pearcy et al. 2004) and *C. hispanica* (*Ch23*, *Ch08*, *Ch01*; Darras et al. 2014). Individual ant DNA was extracted using Chelex extractions (Walsh et al. 1991). Ant legs were crushed and incubated for 90 min at 85 °C in 100 µl of 5 % Chelex with constant agitation. After a 3-min centrifugation at 12,000 rpm, 80 µl of the supernatant was transferred into a 1.5-ml tube. Polymerase chain reaction (PCR) amplifications were carried out in a 15-µl volume, containing 2 µl of genomic DNA, 7.5 µl of Type-it Multiplex PCR Master mix, 0.3 µl MgCl₂ 25 mM, 1.9 µl dH₂O, and 10 µM of each primer's pair (*Ccur99* and *Ch01*: 0.15 µl, *Ccur93*: 0.4 µl, *Ccur11*: 0.3 µl, *Ccur89* and *Ch23*: 0.1 µl, *Ch08*: 0.05 µl). PCR were performed with a MJ Research PTC-200 thermocycler. After an initial denaturing step of 5 min at 95 °C,

the PCR consisted of 28 cycles of 30 s at 95 °C, 90 s at the annealing temperature of 58 °C, and 30 s at 72 °C, followed by a final extension step of 30 min at 60 °C. PCR products were genotyped using an automated Applied Biosystems ABI 3730 capillary sequencer (Applied Biosystems, Foster city, CA, USA). The size of the different alleles was determined using *Peak Scanner version 1.0* analysis software (Applied Biosystems).

Because no queen was found during excavation, the genotypes of each colony queen and her multiple mates were reconstructed from the multilocus offspring genotypes. This is straightforward since colonies of this species are headed by a single queen (Leniaud et al. 2013). Assignment of individuals to each patriline was determined with the maximum-likelihood method implemented in the software *Colony 1.2* (Wang 2004). All individual assignments were double-checked by eye. Overall, 1 soldier and 4 workers that could not unambiguously be assigned to a patriline were removed from the analyses. We inferred the minimum number of fathers contributing to the progeny of each queen (M_p). The effective queen mating frequency ($M_{e,p}$) was calculated following (Nielsen et al. 2003), with standard errors from jackknifing over colonies. We also estimated the non-detection error for each colony (i.e., the probability that two mates bear the same alleles at all loci studied) (Boomsma and Ratnieks 1996) and the non-sampling error (Foster et al. 1999). Both the population-wide non-detection error ($P_{\text{non-detect}} = 0.005 \pm 0.003$) and the probability that we failed to sample a male contributing to at least 5 % of the progeny of each queen ($P_{\text{non-sampling}} = 0.064 \pm 0.034$) were reasonably low; it is therefore unlikely that our data were affected by these potential sources of errors. Paternity skew (the unequal contribution of each father to the offspring) in each colony was quantified according to (Pamilo and Crozier 1996).

To examine whether the distribution of worker and soldier castes deviated from homogeneity across patrilines, we used two statistical approaches. First, we performed Fisher's exact tests, which accommodates the limited sample sizes in a number of patrilines in some colonies (Sokal and Rohlf 1995). The information obtained from all colonies was then combined using the unweighted Z-transform approach (Whitlock 2005) to obtain an overall P value. The strength of the bias in patriline representation between the soldier and worker castes was estimated by measuring the effects sizes, using Cramer's V (0 = no association, 1 = maximum association; Acock and Stavig 1979). Because Cramer's V is based on ratios, data points of zero in contingency tables (i.e., patrilines that were entirely absent in one of the castes) were replaced by 1 (Rheindt et al. 2005). Such a procedure is conservative and reduced the effect size. Second, we applied the statistical analysis by Wiernasz and Cole (2010), corrected for unequal sample

sizes between workers and soldiers genotyped for each colony. Specifically, we quantified the difference in patriline distribution between the two non-reproductive castes as $\Delta_{PD} = \sqrt{(|a_{OW} - a_{EW}| + |a_{OS} - a_{ES}|)^2 + (|b_{OW} - b_{EW}| + |b_{OS} - b_{ES}|)^2 \cdots + (|n_{OW} - n_{EW}| + |n_{OS} - n_{ES}|)^2}$, where letters a to n represent each patriline, n_{OW} and n_{EW} are, respectively, the observed and expected proportions of workers of the n th patriline, and n_{OS} and n_{ES} are the observed and expected proportions of soldiers of the n th patriline. We then generated 10,000 dummy colonies of size N (workers + soldiers) by randomly reassigning the labels 'worker' and 'soldier' to the N individuals from the n patrilines in the focal colony, following the procedure by Holman et al. (2011). We calculated the expected distribution of Δ_{PD} for each colony under the null hypothesis that the proportion of workers and soldiers was homogeneous across all patrilines. For each colony, we then estimated the effect size as the difference between the observed Δ_{PD} and the mean null Δ_{PD} , divided by the standard deviation of the null Δ_{PD} distribution. The value obtained was divided by $\sqrt{M_p}$ to remove the bias associated with the number of patriline in the colony. A positive effect size indicates that the paternity difference between the worker and the soldier caste is greater than expected by chance. All statistical tests and simulations were carried out with the R 2.11.1 statistical software (R Development Core Team 2013).

Results

We determined the genotype of 201 workers ($x \pm SE = 28.71 \pm 1.98$) and 203 soldiers ($x \pm SE = 29.00 \pm 7.43$) from seven colonies, at seven microsatellites loci. The number of alleles at the loci studied ranged from 3 to 11. Consistent with previous studies, worker and soldier genotypes were compatible with single maternity in all colonies. Reconstructing paternal genotypes from worker pedigree, we found that queens in the study population had mated with 6–14 males (Table 1). The arithmetic mean \pm SE absolute mating frequency M_p was 11.00 ± 3.21 . The contribution of each father in offspring production varied in all colonies, with some patrilines contributing up to 10 times more to worker and soldier progeny than other patrilines (Fig. 2). The paternity skew over all colonies ranged from 0.07 to 0.42 and was on average $S \pm SD = 0.23 \pm 0.12$. Due to bias in paternal contribution, the effective mating frequency $M_{e,p}$ was 8.79 ± 2.90 , slightly lower than M_p .

Queen mating frequency in Merzouga appears higher than reported in another population in Amerzgame, where $M_p = 5.7$ and $M_{e,p} = 5.08$ (Leniaud et al. 2013). Both estimates were obtained from genotypes of field workers; but mating frequency in Amerzgame was calculated from a

Table 1 Paternity frequency data and deviation from homogeneity of worker and soldier castes distributions across patriline in the silver ant *C. bombycina*

Colony	Number workers	Number soldiers	M_p	$M_p > 1$	$M_{e,p}$	Fisher P value	Cramer's V heterogeneity	Δ_{PD}	
								T test P value	Standardized effect size
BM01	30	32	13	12	9.27	0.121	0.258	0.320	0.129
BM02	29	34	14	10	9.39	5.9×10^{-6} ***	0.366	0.001***	0.827
BM03	30	32	6	6	4.91	3.2×10^{-3} **	0.354	0.055	0.653
BM04	29	36	6	6	3.92	0.299	0.181	0.177	0.378
BM07	30	12	13	11	11.34	0.120	0.281	0.999	0.922
BM08	24	30	13	13	11.46	0.169	0.255	0.131	0.311
BM09	29	27	12	11	11.25	0.135	0.327	0.451	0.035
BA16	24	22	4	4	3.27	0.010*	0.315	0.019*	1.035

Colonies, number of workers and soldiers genotyped, total number of patriline (M_p), number of patriline represented by two or more workers ($M_p > 1$), and effective mating frequency ($M_{e,p}$) in seven colonies (BM01–BM09) from Merzouga. P values and effect size for deviation from homogeneity of worker and soldier castes distributions across patriline are given for Fisher's exact tests and Δ_{PD} calculations (see "Methods"). Data previously obtained for colony BA16 from Amerzgane (Leniaud et al. 2013) are given for comparison

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

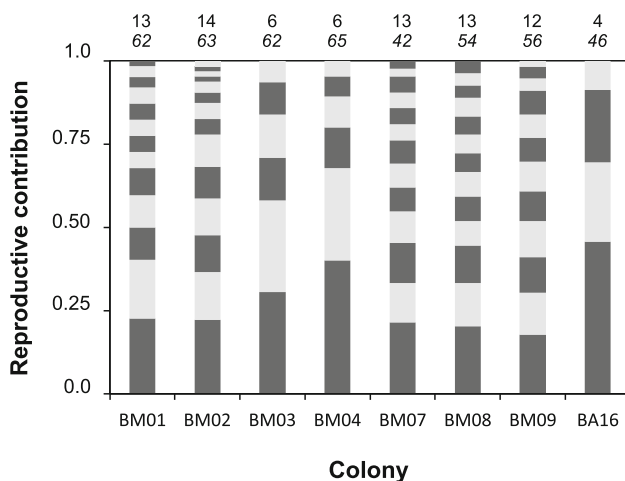


Fig. 2 Frequency distributions of patriline (offspring sired by different males) as estimated from parent–offspring combinations. Patriline are shown by alternate shading patterns, with the total number of patriline and the sample size (*italic*) indicated above the bars for each colony. Data previously obtained for colony BA16 from Amerzgane (Leniaud et al. 2013) are given

much smaller sample size (Amerzgane: $n = 24$ individuals/colony, $N = 10$ colonies; Merzouga: $n = 57.7$ individuals/colony, $N = 7$). To estimate the effect of the sample size on mating frequency, we performed Monte Carlo simulations (Tarpay and Nielsen 2002) on our dataset from Merzouga. We generated 10,000 subsamples of 24 workers per colony and calculated the absolute (M_p) and effective ($M_{e,p}$) mating frequencies. Our simulations show that both estimates were only marginally affected by the reduced sample size ($M_p = 9.21 \pm 0.36$, $M_{e,p} = 8.94 \pm 0.73$), and remained significantly higher than mating frequencies reported for the Amerzgane population. This

supports the view that populations significantly differ in queen mating frequency.

The proportion of workers and soldiers produced per patriline in each colony is shown in Fig. 3. Using Fisher's exact tests, we found statistically significant difference between patriline in their representation in the two castes in 2 out of 7 colonies (Table 1). Colonies BM02 and BM03 displayed an unequal distribution of the worker or soldier castes between patriline; this effect of paternal origin remained significant after adjusting for multiple tests with a step-down Bonferroni correction. Considering all colonies together, there was an overall significant difference between patriline distributions in workers and soldiers (combined P value < 0.001). The effect sizes (Cramer's V) were 0.366 and 0.354 for BM02 and BM03, respectively (mean \pm SE = 0.289 ± 0.060 for the seven colonies from Merzouga), which are medium following Acock and Stavig (1979). When using Wiernasz and Cole (2010) Δ_{PD} approach, deviation from homogeneity of worker and soldier castes distributions across patriline was significant in colony BM02 only (after Bonferroni correction). The unbiased standardized effect size for this colony was 0.827. However, the mean effect size across the seven colonies was 0.465 ± 0.317 , not significantly different from 0 ($P = 0.071$).

Discussion

We used microsatellite DNA analyses of offspring from field colonies to test for a genetic component in soldier caste determination in the silver ant *C. bombycina*. Our data show

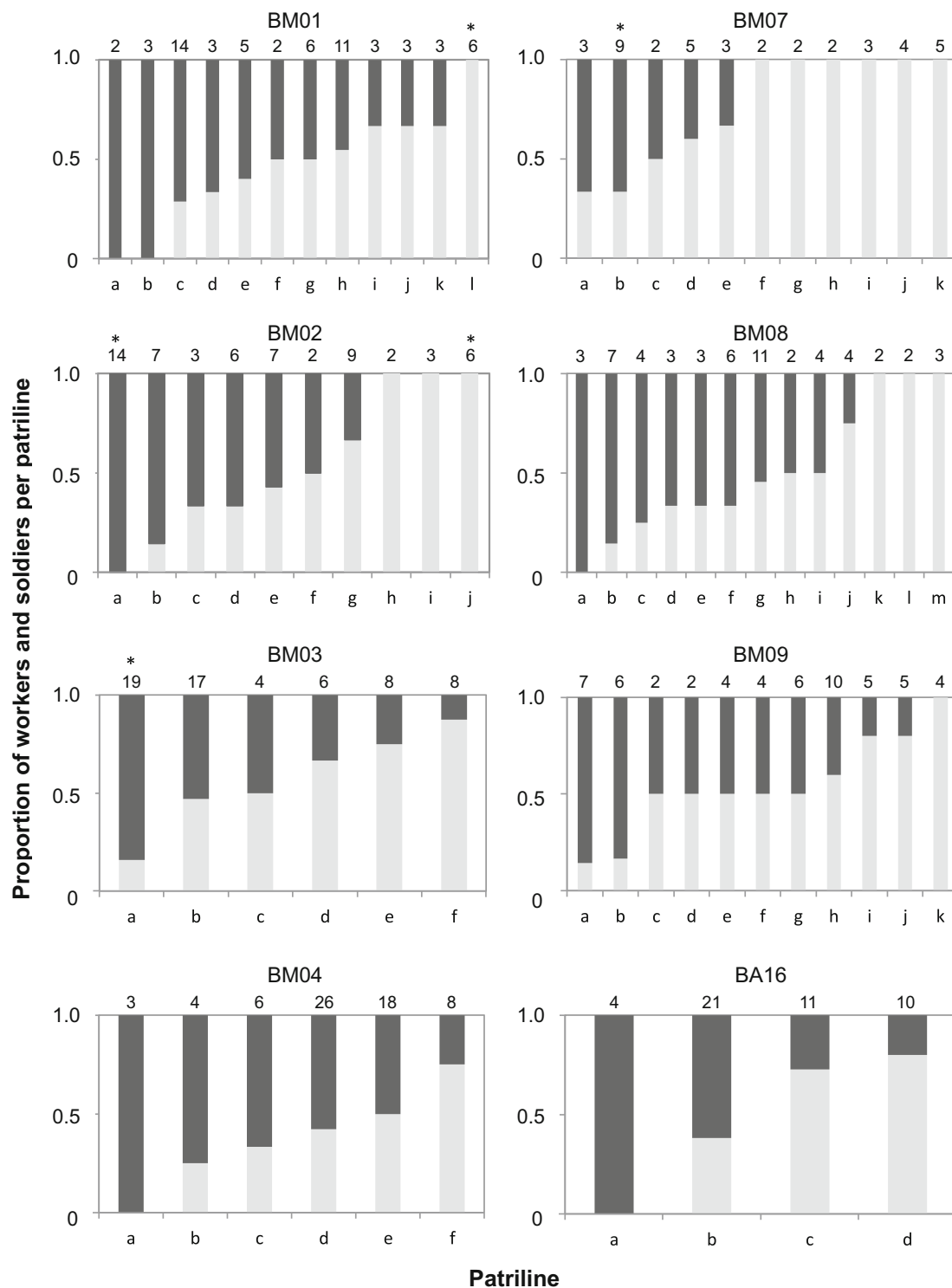


Fig. 3 Proportion of workers (*black*) and soldiers (*grey*) produced per patriline in *Cataglyphis bombycina*. Seven colonies were examined from the population of Merzouga (BM01, $n = 62$; BM02, $n = 63$; BM03, $n = 62$; BM04, $n = 65$; BM07, $n = 42$; BM08, $n = 54$; BM09, $n = 56$). Data previously obtained for colony BA16 from Amerzgane ($n = 46$; Leniaud et al. 2013) are given. The total number

of offspring from each patriline is indicated above the *bars*. Patriline groups represented by less than two workers generate a 100 % ratio and were therefore not shown to avoid misleading interpretation of the data. Asterisk denotes patriline groups for which the worker/soldier ratio is significantly different from expectation under null hypothesis (Binomial test with Bonferroni correction, $P < 0.05$)

that the propensity to develop into the worker or soldier caste varies significantly between patriline in only 2 out of 7 colonies sampled. In contrast, no evidence of genotypic variation in caste propensity was found in the remaining 5 colonies. Moreover, across all colonies, most patriline produced both workers and soldiers. Effect size in each colony was generally low to medium and the mean effect size across all colonies was not significant. Altogether, these results offer support for a moderate genotypic influence to soldier caste determination in the silver ant. Environmental cues, especially trophic factors are thought to be the main drivers of differentiation into either the royal or worker caste, but also different worker forms in social insects (Smith et al. 2008a; Wheeler 1986; but see Schwander et al. 2010 for genotypic effects on caste determination). The influence of environmental effects on developmental programs allows *C. bombycina* to retain plasticity in worker vs. soldier caste determination. Workers tending the larvae may therefore be able to control larval development to keep a favorable ratio of large to small workers, outweighing a potential genetic propensity of some larvae to develop into a particular morph (Hughes and Boomsma 2007; Schmidt et al. 2011). This may allow colonies to respond rapidly to environmental changes, by adaptively adjusting the ratio of each caste in response to colony demography and fluctuations in ecological pressures, such as predation or competition risks, as documented in other ant species (Detrain and Pasteels 1991; Passera et al. 1986; Wilson 1983).

Interestingly enough, we detected a non-random representation of workers and soldiers across patriline in the two colonies BM02 and BM03 from the study population (Merzouga). The genotypic influence to soldier caste determination is confirmed by the combined test over all colonies. These results complement those of Leniaud et al. (2013) showing a significant influence of the genotype on soldier caste determination in colony BA16 from another population (Amerzgane; Table 1). In two colonies, BM03 and BA16, the distribution of patriline between the worker and soldier castes was significantly different in the two statistical tests performed. This result suggests that genotypic effects may partially influence the development of individuals into two discrete castes. Effect size in colonies BM02, BM03 and BA16 were comparable with those found in other studies in ants. Our results of Cramer's V (0.366, 0.354, 0.315, respectively) are in the range of values reported by Chéron et al. (2011), who compared the paternity frequencies between workers and new queens (gynes) in *Cataglyphis cursor*. Likewise, standardized effect sizes calculated from Δ_{PD} (values uncorrected for the number of patriline: 3.09, 1.60, 2.07, respectively) fall in the higher range of those estimated by Holman et al. (2011) who showed that patriline are differently distributed between small and large workers in the leaf-cutting ant *Atta*

colombica. In a similar study on the same species, Evison and Hughes (2011) reported medium-to-large effect sizes using Cohen's f estimate.

It has been argued that genetic differences between castes may stem from temporal variation in sperm use rather than from fertilization bias among ejaculates from different males (Wiernasz and Cole 2010). If different ejaculates remain clumped in the spermatheca, the relative frequency of each patriline may vary over time. This explanation was indeed proposed to account for the biased distribution of patriline between queens and workers in highly polyandrous *Pogonomyrmex* harvester ants (Wiernasz and Cole 2010). However, sperm clumping is expected to be rare in eusocial Hymenoptera. First, it opposes the strong empirical evidence for significant benefits from multiple mating (Crozier and Fjerdingstad 2001; Schmid-Hempel 1998). Sperm admixture results in fitter colonies because it increases genetic diversity in the offspring which, in turn, enhances colony resistance to pathogens (Sherman et al. 1998), as well as division of labor and colony productivity (Crozier and Page 1985; Hughes et al. 2003; Mattila and Seeley 2007; Robinson and Page 1988; Smith et al. 2008a). Thus, sperm clumping after storage and its sequential use over the life spans of queens would thwart genetic advantages of polyandry. Second, and consistent with the argument above, analyses of patriline distributions in offspring sampled over long-time intervals showed that sperm use is generally random in ants (Holman et al. 2011; Kronauer et al. 2006; Stürup et al. 2014) and other social insects (honeybees: Franck et al. 1999; wasps: Goodisman et al. 2007). In line with these results, in all *Cataglyphis* species studied so far (i.e., *C. cursor*, Percy et al. 2009; *C. mauritanica* and *C. altisquamis*, unpubl. data), the number of patriline detected in the work force and in the spermatheca was similar, suggesting that queens do not sequentially use the sperm of their mates. The moderate paternity skew among non-reproductive offspring found in some colonies might therefore stem from variation in sperm contribution of the different fathers, as reported in leaf-cutting ants (Holman et al. 2011). Although one may not completely exclude variation in sperm usage by *C. bombycina* queens over time, this explanation seems unlikely to account for the apparent patriline bias in worker vs. soldier production found in this study.

Pedigree analyses confirmed that all colonies were headed by single, multiply mated queens. We found that queens mate on average with 11 males (range 6–14; $M_{e,p} = 8.79$) in the population of Merzouga. This value is almost twice as high as the 5.7 males (range 3–8; $M_{e,p} = 5.08$) previously reported in the population of Amerzgane (Leniaud et al. 2013). Our data show that the difference in the sample size between both studies does not account for the disparity in the mating frequency found

across studies. Rather, they are consistent with populations significantly differing in queen mating frequency. Biogeographic variations in population-specific queen mating frequencies have been previously documented in ants (Boomsma and Van Der Have 1998; Corley and Fjerdingstad 2011; Sundström 1994; Sundström et al. 1996; Suni and Eldakar 2011; Van der Have et al. 1988) and bees (DeFelice et al. 2014; El-Niweiri and Moritz 2011). Whether such variations rely on demographic, genetic or environmental factors in the silver ant is, however, unknown. Notably, the two *C. bombycina* populations sampled occupy different ecosystems. In Merzouga, the ants live in the extremely dry sand dunes of Saharan desert. Colonies extend several meters deep and are exceptionally populous for the genus. In contrast, Amerzgane is a rugged area along the Atlas Mountains foothills; it receives more rainfall, nourishing wadis and valleys. The population occupies an old wadi facing south, and colonies are much smaller than in Merzouga. These environmental disparities may potentially exert different selection pressures that affect some determinants of the species' life history, including the reproductive strategy (Baird et al. 1986; Stearns 1992).

In summary, this study suggests that soldier caste determination in the silver ant *C. bombycina* is mainly influenced by environmental factors (e.g., maternal effects or differences in rearing conditions). Nevertheless, along with the effect of environmental factors, patriline may have a significant influence on the caste, worker or soldier, an individual will develop into. Whether this genotypic influence on caste determination stems from epistatic, genetic compatibility effects between the parental genomes (Libbrecht and Keller 2013; Schwander and Keller 2008) or from the parental genetic background (i.e., allelic differences between the queens and their mates) remains to be elucidated.

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