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Facultative use of thelytokous parthenogenesis for queen production in the polyandrous ant *Cataglyphis cursor*

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Abstract

The evolutionary paradox of sex remains one of the major debates in evolutionary biology. The study of species capable of both sexual and asexual reproduction can elucidate factors important in the evolution of sex. One such species is the ant *Cataglyphis cursor*, where the queen maximizes the transmission of her genes by producing new queens (gynes) asexually while simultaneously maintaining a genetically diverse workforce via the sexual production of workers. We show that the queen can also produce gynes sexually and may do so to offset the costs of asexual reproduction. We genotyped 235 gynes from 18 colonies and found that half were sexually produced. A few colonies contained both sexually and asexually produced gynes. Although workers in this species can also use thelytoky, we found no evidence of worker production of gynes based on genotypes of 471 workers from the six colonies producing sexual gynes. Gynes are thus mainly, and potentially exclusively, produced by the queen. Simulations of gynes inbreeding level following one to ten generations of automictic thelytoky suggest that the queen switches between or combines thelytoky and sex, which may reduce the costs of inbreeding. This is supported by the relatively small size of inbred gynes in one colony, although we found no relationship between the level of inbreeding and immune parameters. Such facultative use of sex and thelytoky by individual queens contrasts with other known forms of parthenogenesis in ants, which are typically characterized by distinct lineages specializing in one strategy or the other.

Introduction

The evolutionary paradox of sex remains one of the major enduring debates in evolutionary biology despite a long history of research devoted to the topic (Maynard Smith, 1978; Bell, 1982; Otto & Lenormand, 2002). Sex presents a paradox because a female reproducing asexually avoids the two-fold demographic cost of producing males (Maynard Smith, 1978) or, in hermaphrodites, the two-fold cost of genetic dilution during meiosis (Williams, 1975). Thus, under the assumption of all else being equal, any mutant allele coding for asexual reproduction should invade a sexual population. Nonetheless, sex is ubiquitous in animals and plants (Barton & Charlesworth, 1998; West *et al.*, 1999; Engelstadter, 2008), and it is generally accepted that this stems from the benefits of sex in generating genetic diversity (positive effect of recombination), repairing damaged DNA and/or avoiding accumulation of disadvantageous mutations (Bell, 1982; West *et al.*, 1999; Horandl, 2009a).

The theoretical basis underlying the two-fold cost of sex is dependent on two key assumptions: that sexually and asexually produced individuals are equivalent in all

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other characteristics and that sexual and asexual lineages are independent. While these assumptions may have been true at the origin of sex, they are probably less justified when asexuality arises from sexual populations, as is the case typically examined in empirical studies (Simon *et al.*, 2003; Lehtonen *et al.*, 2012). In many species, sexual and asexual reproduction can alternate within a given lineage such as in cyclic parthenogenesis (e.g. Kleiven *et al.*, 1992; Carmona *et al.*, 2009). Moreover, shifts from sexual to asexual reproduction are often associated with changes in life history and/or functional or evolutionary constraints that could influence the relative fitness of sexual and asexual individuals (Engelstadter, 2008; Horandl, 2009b; Lehtonen *et al.*, 2012). For instance, whereas apomixis (thelytoky with no meiosis) maintains the level of heterozygosity, some forms of automixis (meiosis followed by the restoration of diploidy) reduce it (Suomalainen *et al.*, 1987) and thus could lower the fitness of asexuals by exposing them to recessive deleterious alleles (Archetti, 2005; Engelstadter, 2008).

Additional complexities arise when trying to understand the evolution of thelytoky in social insect species, because thelytoky can be used not only by queens but also by workers and, furthermore, can be used to produce either queens or workers. Thelytoky evolved repeatedly in social insects through a mutational route as it does not result from bacterial infection or hybridization process (reviewed in Wenseleers & Van Oystaeyen, 2011; Rabeling & Kronauer, 2012). Thelytoky is restricted to the queen in some species, but in species in which workers have ovaries, it opens the possibility for asexual reproduction by workers and in doing so, the potential for reproductive conflicts (e.g. *Apis mellifera capensis*, Beekman *et al.*, 2009). However, when thelytoky completely replaces sexual reproduction for the production of both workers and queens, colonies are nearly clonal and potential conflicts are essentially nonexistent (conflicts reviewed in Ratnieks *et al.*, 2006; e.g. *Platytyrea punctata*, Hartmann *et al.*, 2005; *Mycocepurus smithii*, Himler *et al.*, 2009). Given that these conflicts are costly (Gobin *et al.*, 2003; Bocher *et al.*, 2008), this may facilitate the maintenance of thelytoky. In other species, thelytoky acts as a complement to sexual reproduction and is used only for producing queens (e.g. *Wasmannia auropunctata*, Foucaud *et al.*, 2007). In this case, sexual reproduction remains a major part of the life cycle and costs linked to mating and producing males remain present. These arguments suggest that the evolutionary selective pressures behind thelytoky can be diverse with the costs and benefits of thelytoky depending on the social context.

In the ant species, *Cataglyphis cursor*, both workers and queens are known to use thelytokous parthenogenesis (Cagniant, 1979; Pearcy *et al.*, 2004a; Clémencet *et al.*, 2008). The queen uses thelytoky to produce gynes (new queens) and sexual reproduction to produce workers (Pearcy *et al.*, 2004a). Because males are still produced (Pearcy & Aron, 2006a), asexual reproduction does not avoid the ecological cost of producing males. However, reproducing asexually does increase the number of genes transmitted by the queen. Indeed, assuming that the mode of reproduction does not affect the sex ratio or the total number of offspring produced, the queen transmits one set of genes via males. In addition, an asexually reproducing queen transmits both sets of genes via clonal gynes, whereas a sexually reproducing queen transmits only one set. Hence, asexually reproducing queens have a 1.5-fold advantage compared to sexually reproducing queens (3 vs. 2 sets of genes transmitted), similar to the cost of gene dilution in hermaphrodite species in the absence of male production (box 3 in Lehtonen *et al.*, 2012). *C. cursor* queens benefit from the advantages of both modes of reproduction (Pearcy *et al.*, 2004a), by transmitting a full complement of genes to the reproductive caste by producing gynes asexually, while simultaneously maintaining the benefits of a genetically diverse workforce by mating with many males and producing workers sexually (Boomsma *et al.*, 2009). However, because *C. cursor* uses automixis with central fusion, thelytoky also comes at the cost of increased inbreeding at each generation (Pearcy & Aron, 2006b). Nonetheless, high levels of inbreeding are expected only if queens use thelytoky consecutively over many generations, because a single generation of sex (assuming panmixis) is sufficient to erase any level of inbreeding. Workers can also use thelytoky and produce both workers and gynes. Worker thelytoky has only been demonstrated in orphaned colonies under laboratory conditions (Cagniant, 1979; Chéron *et al.*, 2011b), but frequent queen death in the field could be a selective force favouring the evolution of worker production of gynes because this allows orphaned colonies to requeen instead of dying (Lenoir & Cagniant, 1986; Pearcy & Aron, 2006b). Whether workers also produce gynes in queenright colonies has never been tested, but it is not expected on theoretical grounds (Wenseleers & Van Oystaeyen, 2011) because workers are on average more related to gynes parthenogenetically produced by the queen ($R = 0.5$) than to those produced by other workers ($R = 0.4$, Fig. 1). Hence, workers should police each other and refrain from reproduction.

Initial studies of *C. cursor* suggested that gynes were predominantly produced thelytokously by the queen (Pearcy *et al.*, 2004a; Pearcy & Aron, 2006b). Recent studies, however, have found that sexual production of gynes is common in another population (Chéron *et al.*, 2011a; Cronin *et al.*, 2012). A difficulty here is that gynes produced sexually by the queen genetically resemble gynes produced asexually by workers: both types of gynes harbour alleles from queen mates, in contrast to gynes produced asexually by the queen. To account for this possibility, we use the term 'sexual'

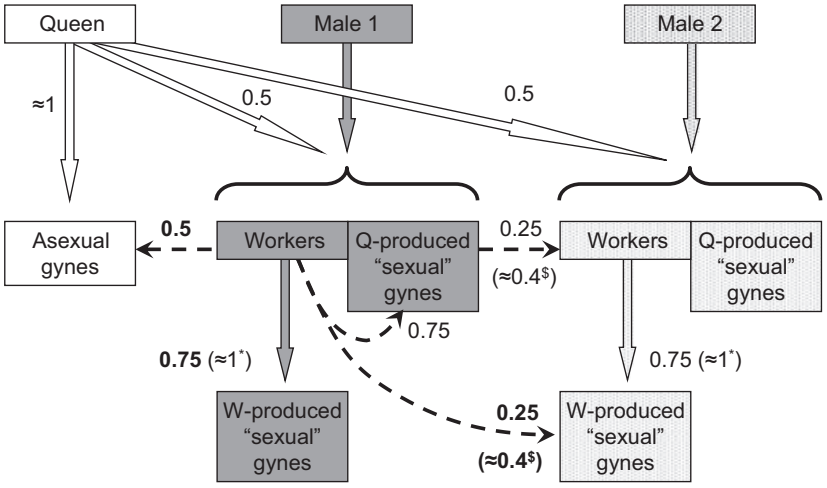

Fig. 1 Parentage of gynes and relatedness pattern. Coloured boxes and arrows represent lineages, and dotted arrows represent relatedness from a focal worker's viewpoint. Gynes may be daughters of the queen produced by thelytoky, in which case they harbour no alleles from any of the queen's mates (asexual gynes). Gynes may also be sexually produced by the queen or thelytokously produced by the sexually produced workers, in which cases they belong to a patriline ('sexual' gynes). Workers of each patriline are most related to gynes from their own patriline (daughters, $R \approx 1$; full-sister workers' daughters, $R = 0.75$; queen-produced full-sisters, $R = 0.75$), but they are also more related to asexual gynes produced by the queen ($R = 0.5$) than to 'sexual' gynes produced by workers on average ($R = 0.44$, both when estimated from Table 2 and when measured among colonies randomly sampled from the same population, Chéron *et al.*, 2011b). Note that the relatedness of queens to their asexually produced gynes is slightly lower than one due to the recombination events that could occur at some loci. * = relatedness to worker's own daughter and $ = average relatedness among workers.

gynes to describe those bearing paternal alleles, whereas those bearing only maternal alleles are referred to as asexual gynes (Fig. 1). We shed light upon the evolution of thelytoky in *C. cursor* (i) by quantifying the relative frequencies of sex and thelytoky in two populations (one being the same as Chéron *et al.*, 2011a and Cronin *et al.*, 2012) and the pervasiveness of worker reproduction and (ii) by comparing the quality of asexual and 'sexual' gynes in terms of inbreeding levels, size and immune defences. Our results show that most, if not all, 'sexual' gynes are queen-produced and that asexual gynes are less inbred than expected under strict thelytoky, demonstrating, for the first time in ants, the alternative use of thelytoky and sex by individual queens for producing new gynes. We suggest that the evolution of thelytoky in *C. cursor* might be understood in the context of fitness-associated sex (FAS) models, where individuals use sex or thelytoky depending on expected fitness returns (Hadany & Otto, 2009).

Materials and methods

Sampling effort

Eighteen colonies were excavated between the 20th of May and the 3rd of June 2007, the time of mating and colony fission (Chéron *et al.*, 2011a), at two localities distant of 9.5 km from each other and located near Perpignan (South of France). We selectively targeted colonies where gynes were present at the nest entrance and collected ten colonies containing 103 queens at Argelès-sur-mer (42.5722508°N, 3.0436708°E; site A2 in Clémencet *et al.*, 2005) and eight colonies containing 144 queens at Saint Cyprien (42.655150°N, 3.033180°E; patch 3 of the seaside transect in Clémencet *et al.*, 2005).

It is sometimes impossible to morphologically distinguish the queen from gynes in *C. cursor*, because gynes often lose their wings before colony fission occurs. The queen was thus identified *a posteriori* by dissecting ovaries (see below). Colonies were transferred to plastic containers as soon as collected and subsequently maintained with regular feeding. From each of the 18 collected colonies, 50–100 workers were removed and placed in 95% ethanol (5 Tris–EDTA) soon after collection. For all but three colonies (75, 122 and 123), the queen and gynes were killed in a field laboratory one or two days after excavation for hemolymph collection and physiological analysis. Thoraces and gasters were frozen at −80 °C for later dissection and DNA extraction in the laboratory. For the three remaining colonies, gynes were kept alive in the laboratory and used for preliminary tests on mating behaviour. These gynes were thus not used for hemolymph collection and were excluded from the analyses of mating status.

Genetic analysis

DNA was extracted from the thorax of queens and gynes and from the head of workers using Qiagen DNA

tissue kit QIAquick 96 and eluted in 150 μL elution buffer. Twelve microsatellite loci were used (Table 1, Pearcy *et al.*, 2004b; Chéron *et al.*, 2011b). PCRs were carried out in a 10-μL volume containing 1 μL DNA solution (10–40 ng of DNA), 200 μM of each dNTP, 100 nM of each primer (except for Ccur65: 250 nM; Ccur99: 1501 nM; Ccur61: 75 nM; Ccur100: 200 nM), 1X Taq buffer (with $MgCl_2$ 1.5 mM final) and 1 unit of Taq DNA polymerase (Q Biogen). Thermocycle conditions were as follows: 10 min at 94 °C followed by 10 amplification cycles with 94 °C for 15 s, 52 °C for 15 s, 72 °C for 30 s, 20 amplification cycles with 89 °C for 15 s, 52 °C for 15 s, 72 °C for 30 s and a final elongation step of 10 min at 72 °C. Four sets of loci were co-amplified (multiplex 1: Ccur26, Ccur46, Ccur76; multiplex 2: Ccur11, Ccur63, Ccur89; multiplex 3: Ccur51, Ccur58, Ccur65, cur99; multiplex 4: Ccur61, Ccur100). Amplification products of two multiplexes (multiplexes 1 and 2 or multiplexes 3 and 4) were loaded together on an ABI Prism 310 sequencer (Applied Biosystems), and allele sizes were estimated using GENESCAN software.

Genetic analyses had two objectives. First, we genotyped all 247 queens and gynes at the 12 loci in order to determine the reproductive mode used for gyne production and their inbreeding level. Second, we conducted a paternity analysis on 475 workers from six colonies that produced more than five 'sexual' gynes in order to assess the origin of 'sexual' gynes (worker- or queen-produced). We initially analysed the workers with six loci only (multiplexes 1 and 2) to determine patrilines. Then, for at least two workers per patriline, the genotype was obtained for the other six loci in order to get the paternal genotype for all 12 loci.

To better estimate background population allelic frequencies, we added to our worker sample of six colonies one worker from the other 12 collected colonies and one worker from 24 neighbouring colonies (12 in each site) and genotyped these for all 12 loci. Background population allelic frequencies were estimated for the two sites using the full data set containing all workers using the software RELATEDNESS 5.0.8, and weighting colonies equally. These allelic frequencies were used to estimate the expected genetic diversity (H_e) and used in subsequent analyses performed with the software RELATEDNESS, MATESOFT and in simulations (see below). All 12 loci used were highly polymorphic with a mean of 10 alleles per population (range 4–20) and a mean expected genetic diversity per population of 0.75 (range 0.42–0.92) (Table 1).

Linkage disequilibrium between each pair of loci was examined at the two sites using exact tests with Genepop 4.1 (Rousset, 2008). We randomly selected a single worker per colony from the six colonies in which many workers were analysed. This reduced data set contained 42 workers (21 for each site), each genotyped at 12 loci. Of the 132 tests of linkage disequilibrium performed between each pair of loci in each site, only six were significant, with P values always higher than 0.009. The number of significant tests is not different from what would be expected by chance given the number of tests performed at a 5% level (6.6). Our loci can therefore be considered independent.

Ratio of asexual to 'sexual' gynes

All queens and gynes were dissected under a binocular microscope to determine the status of their ovaries and spermatheca. They were determined as inseminated when the spermatheca was dark brown and virgin when it was translucent and empty. Individuals that

Table 1 Characteristics of the 12 microsatellite loci for Argelès and St Cyprien populations. *Rt* is the rate of transition from heterozygous to homozygous gynes during parthenogenetic reproduction. The number of informative gynes available for this estimate is given between parentheses and combined the gynes studied in this paper and in Chéron *et al.* (2011b). *Na* and H_e are respectively the number of alleles and expected heterozygosity, and the population-level inbreeding coefficient of Weir & Crockerham (1984) is given for workers and queens, respectively, by F_{is} W and F_{is} Q.

		Argelès				St Cyprien			
Loci	Rt (n)	*Na*	H_e	F_{is} W	F_{is} Q	*Na*	H_e	F_{is} W	F_{is} Q
Ccur11	0.00 (255)	13	0.81	−0.07	−0.06	9	0.78	−0.02	−0.03
Ccur26	0.00 (81)	8	0.67	−0.05	0.00	4	0.42	−0.00	0.1
Ccur46	0.19 (265)	5	0.75	−0.22	0.62	12	0.86	−0.08	0.368*
Ccur51	0.02 (240)	12	0.71	−0.20	0.10	11	0.82	0.04	0.09
Ccur58	0.32 (265)	13	0.78	0.07	0.82**	9	0.82	−0.01	0.51*
Ccur61	0.14 (310)	20	0.92	0.06	0.54**	13	0.85	0.04	0.65*
Ccur63	0.09 (58)	14	0.82	0.16	0.34	8	0.78	0.00	0.43
Ccur65	0.34 (257)	9	0.54	0.26	0.65**	7	0.56	0.23	0.08
Ccur76	0.50 (14)	11	0.82	0.12	0.80***	7	0.74	−0.01	0.48
Ccur89	0.21 (24)	9	0.72	−0.10	0.65**	9	0.69	−0.24	0.37
Ccur99	0.35 (241)	12	0.76	−0.09	0.70***	8	0.82	−0.07	0.50*
Ccur100	0.03 (281)	11	0.82	−0.10	−0.23	10	0.73	0.03	−0.33

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

were inseminated and had fully developed ovaries and unambiguous yellow bodies, indicating prior egg laying, were considered queens, whereas the others were considered as mated gynes.

The reproductive mode used for gyne production was determined by visual inspection of their genotypes. When the genotype of the queen was known (14 colonies), a gyne was determined as 'sexual' if she had alleles not present in the queen (i.e. paternal alleles); otherwise, she was considered as 'asexual', originating from thelytokous reproduction of the queen. A 'sexual' gyne would lack distinctive paternal alleles if her father had the same alleles than the queen at all 12 loci examined, but this was highly improbable in the two populations studied ($P < 10^{-7}$). Indeed, 'sexual' gynes had on average 8.2 (range 5–12) diagnostic paternal alleles on the 12 loci. When the genotype of the queen was unknown (four colonies), two or more gynes were considered asexual if they had identical genotypes at the 12 loci studied, except for loci that had undergone recombination (i.e. one gyne was homozygous at a loci heterozygous in the other gynes).

Quality of asexual and 'sexual' gynes

Using Genepop 4.1 (Rousset, 2008), we estimated the population inbreeding coefficient (F_{is}) of Weir & Crockerham (1984) and tested for a deviation from Hardy–Weinberg equilibrium using exact tests, separately for gynes and workers (randomly taking one individual per colony when more than one was available). We also estimated the level of inbreeding at the individual level from the average number of homozygote loci weighted by the allelic diversity at each locus (homozygosity by loci, hereafter HL), as advised by Aparicio *et al.* (2006). Estimates of HL were obtained for each site from the background allelic frequencies using the software R (R-Development-Core-Team, 2008). These observed estimates of HL were compared with theoretical estimates of HL simulated under either sexual reproduction or one to 10 consecutive generations of thelytoky. These comparisons allowed determination of whether the level of inbreeding observed in asexual gynes was consistent with several consecutive generations of thelytoky or with the frequent alternation of thelytoky and sexual reproduction. Theoretical estimates were obtained through the following simulations (script available upon request to C. Doums): a theoretical population of 1000 individuals was randomly built using the population allelic frequency. The HL of these individuals was computed, yielding the distribution of HL under sexual reproduction and assuming panmixis. We then simulated one to 10 consecutive generations of strict thelytoky by these individuals, assuming that the rate of transition (*Rt*) from heterozygosity to homozygosity was that observed for each locus (Table 1). At each generation, we calculated the HL for each of the 1000 individuals, thus obtaining the simulated distribution of HL after various generations of thelytoky. The 95% confidence interval for each of these distributions was obtained by taking the 50th and 950th values of the distribution. We considered that the observed estimates of HL belonged to a given distribution (sexual or one to 10 consecutive generations of thelytoky) if it was within the confidence interval. We also tested whether the estimated HL from the gynes reflects genome-wide level of inbreeding. To do so, for each individual, we estimated HL twice, once with a subset of six randomly selected loci and once with the remaining six loci. We did this for each gyne and correlated the two estimated HL following Balloux *et al.* (2004) and Haag-Liautard *et al.* (2009). This procedure was repeated 1000 times using the software R (script available upon request to C. Doums). The average of the 1000 simulated correlation coefficients was 0.56 with a 95% confidence interval (0.51–0.61) that did not include zero. This therefore indicates that the variation of homozygosity observed among the gynes reflects at least in part genome-wide heterozygosity and it makes sense to correlate the estimated HL based on the 12 loci with other phenotypic parameters.

Phenotypic parameters of asexual and 'sexual' gynes were measured as follows. First, size was assessed by the tibia length of the right foreleg. The tibia was placed on double-sided tape in a standardized orientation and photographed with a Sony XCD-SX910CR digital camera connected to a binocular microscope. Tibia length was then measured from the digitized picture using NI Vision assistant 7.0 (National Instruments). Second, immune defences were assessed by taking two measurements from the hemolymph: the basic level of phenoloxidase activity (active PO) and the total quantity of phenoloxidase potentially available (total PO), which includes the inactive form of the enzyme (PPO) as well as the active form. Phenoloxidase is one of the most important constitutive effectors of the insect immune system and produces melanin that is used in a range of different immune pathways (Cerenius & Soderhall, 2004). We used the same protocol as Bocher *et al.* (2007) to collect the hemolymph and quantify PO and total PO activity. The level of enzymatic activity was determined as the slope of the linear phase of the reaction (generally between 300 and 600 s after the reaction began) using MICROPLATE Manager 5.2 software.

All statistical analyses comparing asexual and 'sexual' gynes were conducted with the software R using mixed models (proc lme) with colonies considered as a random factor, mode of reproduction (asexual or 'sexual') as a fixed factor or the level of inbreeding (HL) as a covariate. The strong dependency between HL and the reproductive mode did not allow us to enter the two variables simultaneously in the model. The dependent variables tested were tibia length, level of PO and level of total PO. The effect of each factor was tested using a

log-likelihood ratio test comparing the model without the factor of interest and the full model. The assumptions of linear models were tested by a visual check of the residuals of the model. The level of PO and total PO was log-transformed to satisfy homoscedasticity. Genetic and phenotypic data can be found in Dryad.

Origin of 'sexual' gynes

'Sexual' gynes, that is, gynes with alleles from queen mates, can result from either sexual reproduction by the queen or thelytokous parthenogenesis by workers (Fig. 1). It is only possible to distinguish between these two possibilities via examination of recombination events: in gynes that are daughters of workers, some loci may recombine during thelytoky and become homozygous, at paternal and/or maternal allele(s), and such recombinant gynes would no longer be assigned to an existing worker patriline. We considered that a 'sexual' gyne was worker-produced when some loci identified her with a worker patriline whereas other loci were homozygous with either a paternal or maternal allele present in the same worker patriline. We investigated the prevalence of worker reproduction in the six colonies that produced more than five 'sexual' gynes (we also estimated the probability of detecting worker-produced gynes for each of these colonies, see Supporting Information). The queen could be identified following ovarian dissection in only two of the six colonies. In the remaining four colonies, the queen genotype was determined from worker genotypes (and genotypes of asexual gynes when present) using Matesoft 1.0 (Moilanen *et al.*, 2004). Patrilines were determined using Matesoft (Table 2 for sample size per colony). The genetic relatedness among workers was estimated using Relatedness 5.0.8., considering each population as a deme (Queller & Goodnight, 1989), and with confidence intervals obtained by bootstrapping over loci. We finally determined whether the distribution of patrilines in workers was similar to their distribution in 'sexual' gynes using a Fisher's exact test implemented in the software R. This program gives an approximate *P* value for the null hypothesis that fathers contribute equally to workers and gynes.

Table 2 Paternity of workers and 'sexual' gynes in the six colonies producing more than 5 'sexual' gynes. Asexual gynes are not presented because they have no father. The sample size (*N*), the actual number of patrilines (*k*), the effective number of patrilines (*ke3*), the estimate of reproductive skew (*B*) and relatedness (*R*) are given for workers (W) and for 'sexual' gynes (G). The value of *B* in boldface indicates that values are significant even after Bonferroni correction for the number of tests performed. All patrilines detected in gynes were found in workers.

Colony	Caste	*N*	*k*	*Ke3*	*B*	*R* (± SE)
54	W	84	7	5.55	**0.037****	0.39 (0.16)
	G	33	7	6.78	0.003	0.38 (0.12)
73	W	82†	9	4.36	**0.117****	0.62 (0.16)
	G	6	3	3.17	−0.055	0.63 (0.15)
75	W	83	13	7.24	**0.060*****	0.32 (0.08)
	G	9	3	2.84	0.00	0.42 (0.13)
106	W	57	8	6.80	**0.021***	0.38 (0.13)
	G	16	5	4.65	0.008	0.60 (0.13)
110	W	66‡	12	8.65	**0.031*****	0.46 (0.31)
	G	16	5	4.65	0.008	0.52 (0.16)
123	W	99§	5	2.89	**0.145*****	0.44 (0.16)
	G	28	4	4.03	−0.004	0.48 (0.14)

†83 workers were genotyped but one was foreigner and removed from the analysis.
‡67 workers were genotyped but one worker was triploid and removed from the analysis.
§101 workers were genotyped but two workers were foreigners and removed from the analysis.
*$P < 0.05$; **$P < 0.001$; ***$P < 0.0001$.

Results

Ratio of asexual to 'sexual' gynes

Dissections allowed the unambiguous identification of the queen in 10 of 18 colonies, and this identification was confirmed by genetic data. The remaining eight colonies (five from Argelès and three from St Cyprien, Fig. 2) were classified as queenless, as they only contained gynes with poorly developed ovaries with no yellow bodies, suggesting either the loss of the queen during colony sampling or a high frequency of queenless colonies at the time of our sampling. This latter possibility is likely as queen replacement is frequent during colony fission (Chéron *et al.*, 2011a) and the timing of excavations aligns well with the timing of colony fission.

Two gynes (in colonies 73 and 123) and one worker (in colony 110) were triploids, with three alleles at eight loci or more. The DNA of the two gynes was re-extracted and yielded the same genotype, which shows that triploidy did not result from contamination problems and that rare triploid individuals occur in natural populations. These three triploids were removed from all analyses. We also removed three foreign workers (one in colony 73 and two in colony 123).

The genotype of the queen was therefore available for the 10 queenright colonies and was inferred from worker genotypes in four of the queenless colonies. No queens shared multilocus genotypes. Overall, 121 gynes were asexual (51.5%) and 114 were 'sexual'. There was no difference in the percentage of asexual gynes between the two populations (Fisher's exact test $P = 0.59$; Argelès: 49.5%, $N = 97$; St Cyprien: 53.6%, $N = 138$). However, within each population, colonies differed significantly in their ratio of asexual gynes (Fig. 2a, Fisher's exact test: Argelès, $P < 0.0001$; St Cyprien, $P < 0.0001$).

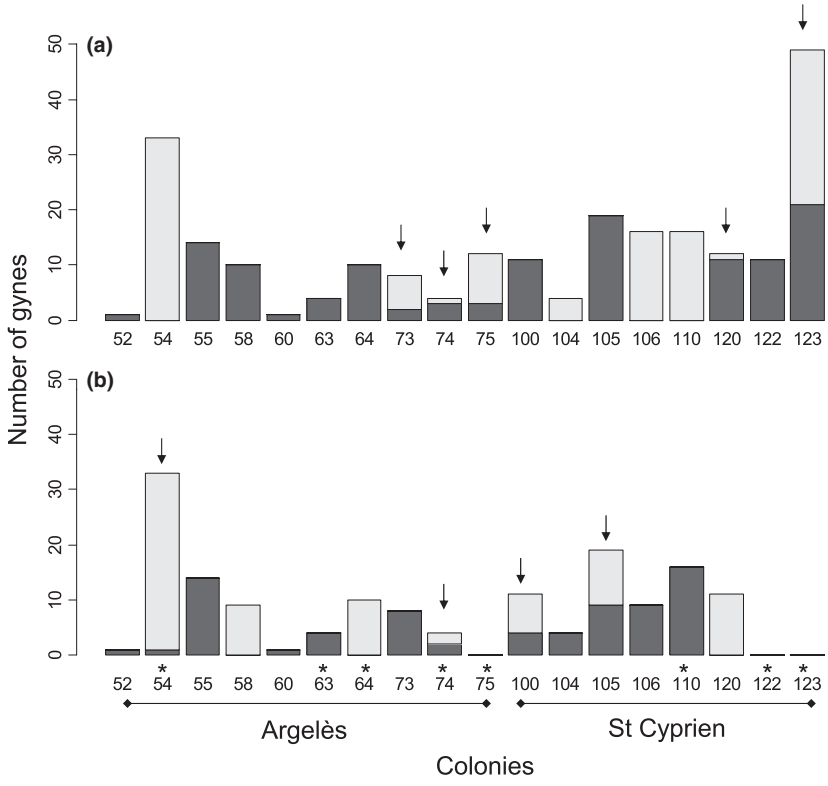

Fig. 2 Frequency distribution of (a) asexual (black) and 'sexual' (grey) gynes and (b) of virgin (black) and mated (grey) gynes. Asterisks indicate queenless colonies. Note that the ratio of mated gynes was not determined for colonies 75, 122 and 123 as these gynes were kept in the laboratory and allowed to mate before dissection. Arrows point to colonies with a mixture of 'sexual'/asexual gynes (a) or mated/unmated gynes (b).

Only five colonies contained a mixture of asexual and 'sexual' gynes (Fig. 2a). Both queenright and queenless colonies produced all three types of combination (only asexual gynes, only 'sexual' gynes or a mixture of both, Fig. 2a). The number of gynes produced was similar in colonies producing only asexual gynes (median = 14, range = 1–19) and in those producing at least one 'sexual' gyne (median = 14, range = 4–49) (Wilcoxon rank test: $W = 25.5$, $P = 0.57$). Colonies producing at least one 'sexual' gyne were headed by a more inbred queen (median = 0.60, range = 0.32–0.91) than colonies producing only asexual gynes (median = 0.36, range = 0.15–0.67), but the difference was not significant (Wilcoxon rank test: $W = 11$, $P = 0.18$).

The percentage of mated gynes was lower in St Cyprien (40.0%) than in Argelès (63.1%) (Fisher's exact test, $P = 0.006$) and varied significantly among colonies in both population (Fisher's exact test: $P < 0.0001$ at both sites) (Fig. 2b). Only four colonies contained both mated and unmated gynes (Fig. 2b). The presence of wings was a good predictor of mating status as most virgins still had their wings (83.5%), whereas most mated gynes had lost them (84%). At the colony level, there was no correlation between the ratios of mated to unmated gynes and of asexual to 'sexual' gynes (Spearman's rank correlation: $z = 0.83$, $P = 0.40$, $N = 15$). We therefore found no evidence that asexual and 'sexual' gynes differed in age (i.e. that one type was produced before the other) at the population level, but this could not be tested within colonies.

Quality of asexual and 'sexual' gynes

At the population level, significant deviation from Hardy–Weinberg equilibrium was found in both populations for gynes (Table 1, Argelès: $\chi^2 = 89.99$, d.f. = 24, $P < 0.0001$, St Cyprien: $\chi^2 = 50.30$, d.f. = 24, $P = 0.0013$), but not for workers (Table 1, Argelès: $\chi^2 = 26.58$, d.f. = 24, $P = 0.32$, St Cyprien: $\chi^2 = 20.83$, d.f. = 24, $P = 0.65$), as already observed in Pearcy *et al.* (2004a). At the individual level, the level of homozygosity per locus (HL) was higher in asexual than in 'sexual' gynes in both populations (average value (range) in Argelès: asexual = 0.63 (0.40–0.75); 'sexual' = 0.14 (0–0.41); St Cyprien: asexual = 0.53 (0.15–0.91); 'sexual' = 0.25 (0–0.49); Fig. 3). This difference was statistically significant (mixed model with colony as random factors and reproductive mode as fixed factor; effect of reproductive mode: *L-ratio* = 197.65, $P < 0.0001$).

In agreement with these results, the mean colony HL of asexual gynes from Argelès (0.63) and St Cyprien (0.53) was lower than as expected under more than nine generations of thelytoky (below the range of the simulated distributions) except in one colony (in St Cyprien) (Fig. 3). This indicates that colonies alternate sexual and thelytokous reproduction, even though

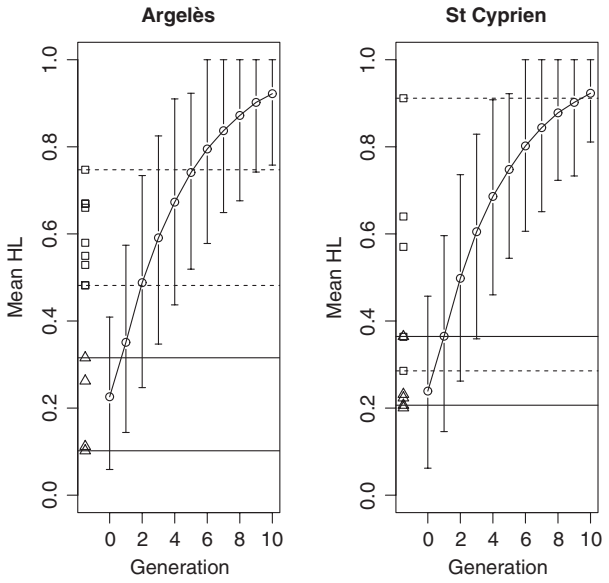

Fig. 3 Observed values of mean level of individual homozygosity by loci (HL) per colony for asexual gynes (squares) and 'sexual' gynes (triangles), and estimated values of HL (circles) for the two populations studied. Horizontal lines represent the upper and lower levels of observed HL for asexual gynes (dashed lines) and 'sexual' gynes (solid lines). The curve and 95% confidence interval estimated over 1000 simulations is given assuming sexual reproduction (generation 0) or 1–10 generations of thelytoky. Simulations are based on the population allelic frequencies and transition probability from the heterozygous to homozygous state (*Rt*) observed in the 12 loci studied. Panmixis is assumed for sexual reproduction (generation 0).

most colonies exhibit only one or the other during any one breeding cycle.

The HL level and the mode of reproduction did not affect the phenoloxidase activity, a key enzyme involved in different pathways of the immune system (Cerenius & Soderhall, 2004) or the size of gynes (Table 3). We also carried out this analysis on colony 123, the only colony producing both enough asexual and enough 'sexual' gynes to allow a colony-level analysis. The size of gynes significantly decreased with increased inbreeding (linear model including the effect of inbreeding both as a factor and as a continuous variable to account for multiple values of Y on X; regression effect: $F_{1,26} = 13.94$; $P < 0.001$; Fig. 4). Because this colony was kept in the laboratory, we could not perform the same analysis on immune parameters.

Origin of 'sexual' gynes

We assessed whether 'sexual' gynes were produced by queens or workers using six colonies that produced more than five 'sexual' gynes each. Fifty-nine patrilines were detected. Five contained only one worker that differed by only one locus from the genetically closest patrilines. For three of these patrilines, the allele at the discriminant locus differed by only two base pairs from the allele of the closest patriline, suggestive of mutation events or PCR errors. In the two other cases, the discriminant locus was homozygous for an allele heterozygous in the closest patriline and could therefore be explained by a parthenogenetic reproduction of a worker of the closest patriline. These discriminant loci were removed, and the five individuals were assigned to their closest patrilines for the subsequent analysis. Nine other patrilines also contained a single worker that differed from other patrilines by more than three loci and were therefore considered as true patrilines. We found no evidence of asexual production of workers by the queen, whereas after correcting for the probability of nondetection of paternal alleles, Pearcy *et al.* (2004a) found that 2.5% of workers were asexually produced.

On average, we identified 9.0 patrilines per colony in workers and 4.5 in 'sexual' gynes (Table 2). All patrilines observed in 'sexual' gynes were also present in workers (Fig. 5). The average effective paternity ($k_{e3} = 5.91$) fell significantly below the observed number of fathers for workers ($k = 9$; paired Wilcoxon test; $Z = 2.2$, $P = 0.028$), whereas it was similar for gynes ($k_{e3} = 4.35$, $k = 4.5$; paired Wilcoxon test; $Z = 1.36$, $P = 0.17$) (Table 2). In agreement with these results, in each colony the paternity bias was significantly greater from zero for workers, but not for 'sexual' gynes (Table 2). The pattern of patriline distribution differed significantly between workers and 'sexual' gynes in two of six colonies (Fig. 5). In these two colonies, some males sired a disproportionately high number of gynes compared to workers. The number of patrilines in these colonies (8 and 5) was similar to the average number of patrilines observed in brood (Pearcy *et al.*, 2004a). Hence, there is no indication that a recent queen turnover could have generated the observed difference in patriline distribution between workers and gynes.

None of the genotypes of 'sexual' gynes could be explained by parthenogenetic reproduction by workers, whereas two workers could have been parthenogenetically produced by other workers (one in colony 110 and one in colony 106). For each gyne, the probability of detecting at least one recombination event if she was worker-produced was high, with an average of 0.83 (col54: 0.89; col73: 0.79; col75: 0.89; col106: 0.79; col110: 0.80; col123: 0.83, see Supporting Information); hence, gynes were predominantly, if not entirely, produced by the queen. However, the fact that two workers could have been produced parthenogenetically by other workers (see above) and our detection power suggest that low frequency of gyne production by workers may occur in natural queen-right colonies.

Table 3 Effect of mode of reproduction and inbreeding (HL) on the level (Vmax) of phenoloxidase activity (PO = active form and totPO = the inactive and active form) and size (tibia of the foreleg). Mean and standard error of the mean are given for asexual and 'sexual' gynes. The effect of the mode of reproduction or the level of inbreeding (fixed effect) was tested using a linear mixed effect model (on log-transformed data for PO and totPO) with colonies as random factor and worker size as covariate. The effect of the mode of reproduction or the inbreeding level was assessed using a likelihood ratio test comparing the model with and without the effect. Both the likelihood ratio value (*L-test*) and their associated *P* values are reported.

	Mean ± SE		Mode of reproduction		Inbreeding (HL)	
Variable	Asexual	'Sexual'	*L-ratio*	*P* value	*L-ratio*	*P* value
Log(totPO)	2.90 ± 0.09	2.78 ± 0.10	0.51	0.47	0.11	0.73
Log(PO)	1.96 ± 0.13	1.54 ± 0.14	0.40	0.53	0.65	0.42
Size	2.08 ± 0.01	2.02 ± 0.01	0.24	0.63	2.86	0.09

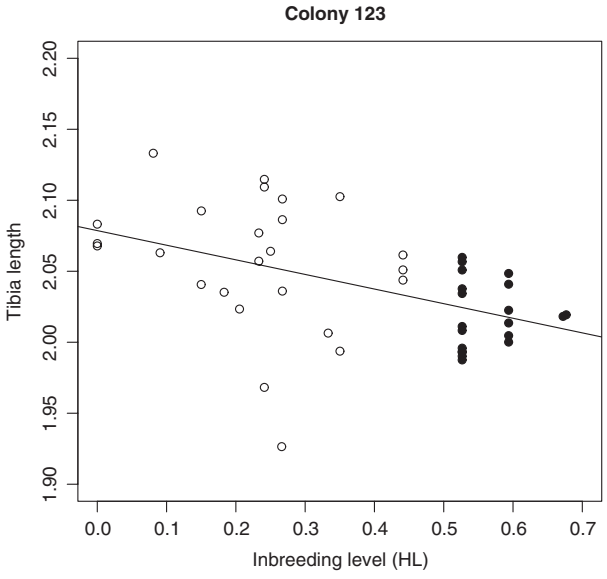

Fig. 4 Effect of inbreeding on the size of gynes (tibia length of the right foreleg) in colony 123, where enough asexual and 'sexual' gynes were available. The level of inbreeding was estimated by the homozygosity level (HL) with zero and one representing respectively a completely heterozygote and homozygote individual. The regression line was drawn from the linear model including the effect of inbreeding both as a factor and as a continuous variable to account for multiple values of Y on X. Filled and empty circles correspond to asexual and 'sexual' gynes, respectively.

Discussion

In the ant species, *Cataglyphis cursor*, both workers and queens are known to use thelytokous parthenogenesis (Cagniant, 1979; Pearcy *et al.*, 2004a; Clémencet *et al.*, 2008), so that 'sexual' gynes (containing paternal alleles) could be produced by queens or workers. Indeed, gynes produced sexually by the queen genetically resemble gynes produced asexually by workers: both types of gynes harbour alleles from queen mates, in contrast to gynes produced asexually by the queen. Our results indicate that most, if not all, 'sexual' gynes were queen-produced, suggesting that thelytoky evolved in this species mainly through selective pressures acting on the queen. The frequency of 'sexual' gynes was high (~50%) in the two populations studied. This contrasts with another population studied by Pearcy *et al.* (2004a) in which nearly all gynes were asexually produced, although Pearcy & Aron (2006b) concluded that sexual reproduction should be frequent (more than 60%) based on population inbreeding values (F_{is}) of gynes. The individual levels of inbreeding (HL) of asexual gynes in our study reveal that thelytoky is rarely employed for more than ten consecutive generations. Given that most 'sexual' gynes were produced by the queen, and that a few queenright colonies produced both types of gynes, we can conclude that the reproductive strategy of *C. cursor* is one which alternates, and at times combines, sexual and thelytokous production of gynes. The ability for an individual queen to facultatively employ sexual and asexual reproduction to produce a given caste (gynes) has never been reported in ants, although the occurrence of mixed 'sexual' and asexual queens in some populations of the fungus-growing ant *M. smithii* (Rabeling *et al.*, 2011) and of *P. punctata* (Kellner *et al.*, 2012) is suggestive. The pattern of variation in the reproductive system of *C. cursor* therefore appears quite different from most other thelytokous social hymenoptera in which asexual lineages reproduce strictly by thelytoky (obligate parthenogenesis), and are therefore clearly separated from sexual lineages, at least for the production of queens (Foucaud *et al.*, 2007; Dobata *et al.*, 2009).

Automictic thelytoky with central fusion is associated with a strong decrease in heterozygosity in each generation (Suomalainen *et al.*, 1987; Pearcy & Aron, 2006b). As expected with this mode of thelytoky, asexual gynes were significantly more inbred than sexual gynes. We detected no strong negative effect of inbreeding on the size or immune defences of individuals over all colonies, although inbred gynes were significantly smaller in the only colony containing both enough 'sexual' and enough asexual gynes to conduct a colony-level test. It is likely that this smaller size of inbred queens translates into lower colony growth and

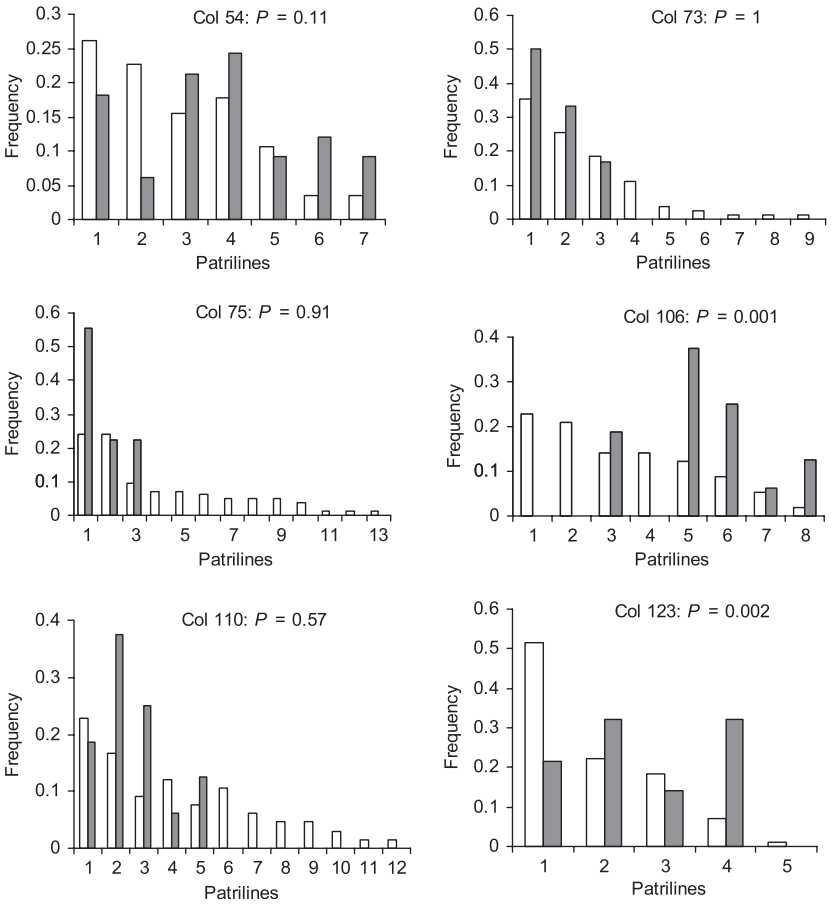

Fig. 5 Patriline distributions in workers (white) and 'sexual' gynes (black) in the six colonies producing more than five 'sexual' gynes. The *P* values are the probability that males contribute equally to workers and gynes estimated using an approximate exact test (see Materials and methods).

survival, as individual size often correlates with fecundity in insects (Honek, 1993), including *C. cursor* (Clémencet *et al.*, 2008). Other studies have demonstrated a negative impact of inbreeding on immune defences in social Hymenoptera (Gerloff & Schmid-Hempel, 2005; Haag-Liautard *et al.*, 2009; Vitikainen & Sundström, 2011). In contrast to our study, inbreeding decreased queen immune defences, but not queen size, in the ant *Formica exsecta* (Vitikainen & Sundström, 2011). It would be informative to examine additional components of the immune system to generalize the effect of inbreeding. The effect of inbreeding depression is generally thought to be minor because deleterious recessive alleles are purged via haploid males in the haplodiploid sex determination system, in which individuals that are homozygous at the sex locus develop into males (reviewed in Werren (1993)). However, this generalization has been questioned because genes coding for female-specific traits are not expressed in males (e.g. Gräff *et al.*, 2007). As a result, they are not purged in haploid males and remain susceptible to inbreeding depression (Henter, 2003). High rates of thelytoky could also purge recessive deleterious mutations and decrease inbreeding depression, as observed for instance in some highly selfing species (Doums *et al.*, 1996; Byers & Waller, 1999). However, the extent of purging depends on many population and genetic parameters, making general prediction difficult (Byers & Waller, 1999). Analysing additional female-specific traits and colony-level parameters in *C. cursor* would be necessary to properly quantify inbreeding depression.

A major additional cost of inbreeding in Hymenoptera is the production of diploid males associated with the single-locus sex determination system (van Wilgenburg *et al.*, 2006). This is because diploids that are homozygous at the sex locus develop into males. These individuals are generally not viable (Cook & Crozier, 1995) and often removed at early brood stages such as in thelytokous honey bees (Goudie *et al.*, 2012). In few species, however, diploid males produce viable diploid sperm that results in the production of sterile triploid offspring (see Table 1 in Darvill *et al.*, 2012). Diploid males thus represent an important cost of inbreeding, both as wasted parental investment and as sterile mates (Whitehorn *et al.*, 2009). Whereas Pearcy *et al.* (2009) found no diploid males in their study population of *C. cursor*, we found viable diploid males in our two study populations and demonstrated that they can mate and father triploid workers (Doums C. unpub. results). This is further validated by the presence of two triploid

gynes and one triploid worker in this study. Diploid male production could therefore be one of the factors constraining the fixation of thelytoky in this species.

Our data indicate that 'sexual' gynes are mainly, if not entirely, produced by the queen. This is contrary to what was previously assumed (Pearcy & Aron, 2006b) and evidence against the hypotheses that thelytoky could have evolved in workers to increase colony life span under frequent queen mortality (Lenoir & Cagniant, 1986; Pearcy & Aron, 2006b). Queen production of 'sexual' gynes is also supported by the fact that colonies without the queen did not produce more 'sexual' gynes than queenright colonies (Fig. 2). Nonetheless, our results cannot completely rule out the possibility of a low frequency of worker cheating in the form of direct production of 'sexual' gynes, or of worker favouritism of queen-produced 'sexual' gynes belonging to the same patriline. Even though workers are expected to favour asexual gynes at a colony level, individual selection could still favour the evolution of rare cheating worker lineages, because workers are on average more related to gynes produced by full-sister workers ($R = 0.75$) than by the queen (Fig. 1). This pattern of conflict is thus reminiscent of the conflict over male production in polyandrous species (Ratnieks, 1988; Wenseleers & Ratnieks, 2006). The observation that two workers were asexually produced by workers suggests that workers may at times attempt reproduction even in the presence of the queen. While this could represent a nonadaptive behaviour, as workers gain little by producing workers, it could also be evidence of unsuccessful attempts at cheating. These attempts fail to produce queens either because worker-laid eggs are less likely to develop into the queen caste due to maternal effects (Schwander *et al.*, 2008) and/or because of worker policing. In queenright colonies, a difference in patriline distribution between workers and 'sexual' gynes was detected in two of the six colonies studied (Fig. 5), in agreement with the pattern previously observed in orphaned colonies (Chéron *et al.*, 2011b). It would be tempting to conclude that this also supports the existence of rare cheating patrilines (e.g. Hughes & Boomsma, 2008; Schwander *et al.*, 2010). However, given that we compared adult workers and young gynes, we cannot exclude potential survival differences among patrilines that could lead to the same pattern (Holzer *et al.*, 2006). In addition, any use of thelytoky by *C. cursor* workers would be rare as it was not detected in our study; hence, it would be markedly different from Cape honey bees, where worker reproduction is timed with gyne production (Beekman *et al.*, 2009) and 59% of gynes are worker-produced (Jordan *et al.*, 2008).

From these arguments, we can surmise that thelytoky in *C. cursor* most likely arose in queens by raising their level of gene transmission (avoidance of the genetic cost of sex), and this advantage could be augmented once thelytoky evolved by a decrease in potential conflicts among worker patrilines. Indeed, based on genetic relatedness only, workers should prefer to rear asexual gynes when relatedness to 'sexual' gynes is lower than 0.5, which was the case in five of six colonies in our study. However, these colonies nevertheless produced more than five 'sexual' gynes each ($0.32 < R < 0.46$, Table 2), suggesting that factors other than relatedness are also important.

Multiple mating appears to be ancestral in *Cataglyphis*, with seven of eight species studied so far being highly polyandrous. Thelytoky, on the other hand, has been unambiguously demonstrated in the field in four of six studied species but probably evolved independently only twice (Pearcy *et al.*, 2004a; Timmermans *et al.*, 2008, 2010; Leniaud *et al.*, 2011, 2012; Eyer *et al.*, 2013). It is therefore likely that, when thelytoky evolved in *C. cursor*, the level of polyandry was similar to that found in present populations (lower than 0.5; Pearcy *et al.*, 2004a; Chéron *et al.*, 2011b), favouring the rearing of asexual gynes produced by the queen. Indeed when the queen mates with more than two males, workers are more related to asexual gynes produced by the queen (0.5) than to 'sexual' gynes produced by other workers (< 0.5).

Given the above benefits of thelytokous gyne production by the queen, why do *C. cursor* queens alternate and/or combine thelytoky with sexual reproduction? Our data suggest that the regular use of sex in *C. cursor* serves to revitalize lineages suffering from inbreeding depression, but this may not be the sole advantage. While we might argue that the ratio observed might not be optimal if populations are not at equilibrium and that the system might finally evolve towards complete thelytoky, theoretical models show that facultative thelytoky might be optimal in some situations (Hadany & Otto, 2007). Thelytoky may be advantageous in a stable environment when the genotype is well suited to that environment. An individual in such circumstances may favour the use of thelytoky, whereas a poorly adapted individual could switch to sex. This concept underlies the 'abandon-ship' hypothesis, which predicts that reproductive strategy may be subject to an individual-quality contingent switch (fitness-associated sex or FAS, Hadany & Otto, 2009). Indeed, in species using facultative or cyclic parthenogenesis, it is often during periods of stress that sexual reproduction is preferred (Otto, 2009). Sex can be associated with a more resistant life stage (*Daphnia*, Kleiven *et al.*, 1992; rotifer, Carmona *et al.*, 2009), a host immune response (nematode *Strongyloides ratti*, West *et al.*, 2001) or a lower fitness (*Aspergillus nidulans*, Schoustra *et al.*, 2010). Clearly, stress can induce sex and this may be the case in *C. cursor*. Sex may be preferred by queens in less-fit colonies and by inbred or otherwise stressed queens. Our result showed that queens producing 'sexual' gynes were indeed more

inbred than those producing asexual gynes, but the trend was not significant in our limited data set. More investigations are needed to test this hypothesis.

Finally, it is important to note that the observed ratio of 'sexual' to asexual gynes might not be optimal from the queen's perspective. Workers may potentially modify this ratio if they can identify the two types of gynes at the brood stage (Aron *et al.*, 2011). Furthermore, males may promote the production of 'sexual' gynes because it is the only way for them to transmit genes in queenright colonies. This could result in an intersexual conflict, but the role males may play in the evolution of mating and reproductive strategies remains largely unexplored in this species.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data S1 estimation of the probability of detecting worker-produced gynes.

Figure S1 The probability of detecting worker produced gynes is given for each colony (curve) as a function of the assumed frequency of worker reproduction.

Data deposited at Dryad: doi:10.5061/dryad.5488t

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