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Sperm production characteristics vary with level of sperm competition in *Cataglyphis* desert ants

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Summary

1. Under polyandry, males are selected to produce more competitive ejaculates. Theoretical models have explored how the mechanism of sperm competition drives males to partition investment within an ejaculate between sperm quantity and quality. The raffle-based competition model predicts that increased level of sperm competition selects for larger numbers of sperm in ejaculates. Sperm competition is also thought to promote the evolution of longer sperm, because longer sperm could be faster.

2. In eusocial Hymenoptera, the mating system imposes unique selective pressures on male ejaculates. Males are short lived; they reach adulthood with a finite amount of spermatozoa, and they mate typically with a single or a few females and die. The actual number of spermatozoa stored in their accessory testes at emergence is thus a reliable measure of total investment into sperm production. In a comparative study of 15 species of *Cataglyphis* desert ants, we used phylogenetically controlled analyses to investigate relationships between levels of sperm competition, sperm production and sperm length. We measured sperm production by quantifying the number of spermatozoa present in testes, instead of using a proxy measure such as size of testes. Multiple queen mating is the ancestral state in the genus but reduction in mating frequency evolved secondarily and independently in some clades, providing a unique opportunity to examine how reduction from multiple to single mating influences sperm traits.

3. Our results provide phylogenetically robust evidence that species experiencing greater levels of sperm competition produce more sperm. After controlling for male size, investment in sperm production decreases significantly according to the sequence obligatory multiple queen mating > multiple–single queen mating > single–double queen mating. Furthermore, the number of spermatozoa produced per male decreases significantly with reduction in paternity frequency for each species. In contrast, neither sperm length nor male size was significantly associated with the mating system classes or the number of patrilines.

4. Our measures of sperm number provide the first direct evidence that sperm production covaries with the level of sperm competition in a eusocial insect. Given the reversal from multiple to single mating in *Cataglyphis*, our comparative analysis also shows convincingly that reduction in sperm competition influences sperm traits.

Key-words: ants, *Cataglyphis*, raffle competition, sperm length, sperm numbers

Introduction

Sperm competition is an important force in the evolution of male reproductive biology and imposes directional selection on sperm traits that enhance fertilization success (Parker 1970a; Birkhead & Møller 1998; Simmons 2001; Birkhead, Hosken & Pitnick 2009). Comparative analyses in a wide range of taxa show that males of species experiencing intense sperm competition, relative to species with weak or no sperm competition, usually produce more sperm to out-compete those of rivals (i.e. fair raffle competition; Parker 1970a; Møller & Briskie 1995; Parker *et al.*

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1996; Hosken & Ward 2001; Ramm & Stockley 2009; Firman, Klemme & Simmons 2013), longer sperm cells that swim faster than shorter ones (Gage 1994; but see Stockley *et al*. 1997; Gage & Freckleton 2003; Immler & Birkhead 2007; Pizzari & Parker 2009; Simpson *et al*. 2014; Bennison *et al*. 2015), and a more uniform sperm morphology that corresponds to an optimal sperm phenotype (Calhim, Immler & Birkhead 2007; Immler, Calhim & Birkhead 2008; Kleven *et al*. 2008; Lifjeld *et al*. 2010; Fitzpatrick & Baer 2011).

Single mating (monogamy) is ancestral in eusocial Hymenoptera (Hughes *et al*. 2008; Boomsma 2009, 2013). However, multiple mating by queens (polyandry) has evolved secondarily in several taxa, most likely driven by the benefits related to enhanced genetic diversity among workers (Boomsma & Ratnieks 1996; Boomsma, Kronauer & Pedersen 2009; Mattila, Reeve & Smith 2012). This secondary evolution of polyandry entails the appearance of sperm competition in the queen genital tract. Yet, the effect of sperm competition on sperm production and size remains only marginally investigated. This is surprising given that the mating system of bees, wasps and ants imposes unique selective pressures on male ejaculates that are rarely, if ever, found in other animals (reviewed in Boomsma & Ratnieks 1996; Baer 2003, 2005; Boomsma, Baer & Heinze 2005; Boomsma, Kronauer & Pedersen 2009; Baer 2011). First, mating typically involves obligate partner commitment for life. Both males and queens mate during a single nuptial flight, at the beginning of adult life. During this short time window, queens mate with one or several males and store a lifetime supply of sperm in their spermatheca. They never remate later in life, even though they may survive and reproduce for decades. Thus, the time between insemination and fertilization may be very long. Males, on the other hand, have usually completed their lifetime spermatogenesis when they reach sexual maturity and do not subsequently increase their supply of sperm. They die shortly after copulation, by exhaustion and/or predation, but persist posthumously as spermatozoa stored in the queen spermatheca. Both sperm limitation and short life expectancy restrict male mating opportunities to a single or a few females (but see Boomsma, Baer & Heinze 2005 for multiple mating by males in some species). Secondly, males of Hymenoptera being haploid, their germinal cells do not undergo meiosis and all sperm cells produced by a given male are genetically identical. The clonal nature of hymenopteran sperm removes intra-ejaculate sperm competition, thereby relaxing selection at the level of individual sperm cells (Sivinski 1984). Thirdly, males usually deposit their ejaculate in the *bursa copulatrix,* an organ located before the spermatheca in the queen genital tract. At this stage, sperm may spend up to several hours in the *bursa* before reaching the spermatheca (Baer 2011). In species where queens are multiply inseminated, this primary storage might result in male ejaculates competing for access to the spermatheca. So far, there is no evidence for sperm displacement in eusocial Hymenoptera. However, recent work showed that seminal fluid proteins from the accessory glands can play a key role in sperm competition by reducing survival of rival male sperm (den Boer, Baer & Boomsma 2010; Den Boer *et al*. 2015). Once the sperm enters the spermatheca, female secretions silence these hostile ejaculate components. Consistent with the concept of fair raffle sperm competition, genetic studies in ants have shown that paternity skew is directly related to the number of spermatozoa each male achieves to get stored in the spermatheca (Schlüns *et al*. 2003; Holman *et al*. 2011). Male fitness is function of their contribution to the production of daughter queens only, since workers are usually sterile and do not reproduce (Crozier & Pamilo 1996). Altogether, these life-history characteristics imply that sperm competition in ants, bees and wasps takes place during a very restricted time window, early in the adult life of both sexes, and that male ejaculates are under strong selection to maximize sperm transfer and sperm storage in the female spermatheca.

So far, a single comparative analysis examined the effects of sperm competition on the size of male sexual organs in eusocial Hymenoptera. In attine fungus-growing ants, males of species in which queens evolved multiple mating have increased accessory testis size (the morphological structure in which mature males store their sperm prior to ejaculation) (Baer & Boomsma 2004). A few studies also focused on the effect of sperm competition on sperm length, but their results remain ambiguous. A higher paternity frequency selects for longer sperm in social bees (Baer *et al*. 2003), but this effect is weak in attine fungus-growing ants (Baer *et al*. 2009). Recently, a comparative study of 27 species of eusocial bees and ants showed that variation in sperm length is negatively associated with paternity frequencies (Fitzpatrick & Baer 2011). This is consistent with the prediction that sperm competition exerts stabilizing selection pressure favouring sperm lengths close to a species-specific optimum.

Here, we assessed the interaction between levels of sperm competition and sperm traits in *Cataglyphis* desert ants. Species-specific genetic analyses showed that mating frequency greatly varies (Lenoir *et al*. 2009; Leniaud, Darras & Aron 2010), providing an almost 10-fold variation in the level of polyandry across species. Furthermore, queens of polyandrous species can copulate with several males over a short time window (e.g. up to eight different males within less than an hour in *C. cursor*; Pearcy *et al*. 2009), and competition between ejaculates for storage in the spermatheca is expected to be particularly intense. Recent phylogenetic analyses showed that multiple queen mating is the ancestral state in *Cataglyphis* and has been maintained in most species; however, reduction in mating frequency appeared secondarily and independently in some clades (Leniaud, Darras & Aron 2010; Fig. 1). These reproductive features make *Cataglyphis* ants well suited for studying how sperm traits are shaped as a function of levels of sperm competition across closely related species. In particular, they provide a unique opportunity to examine how

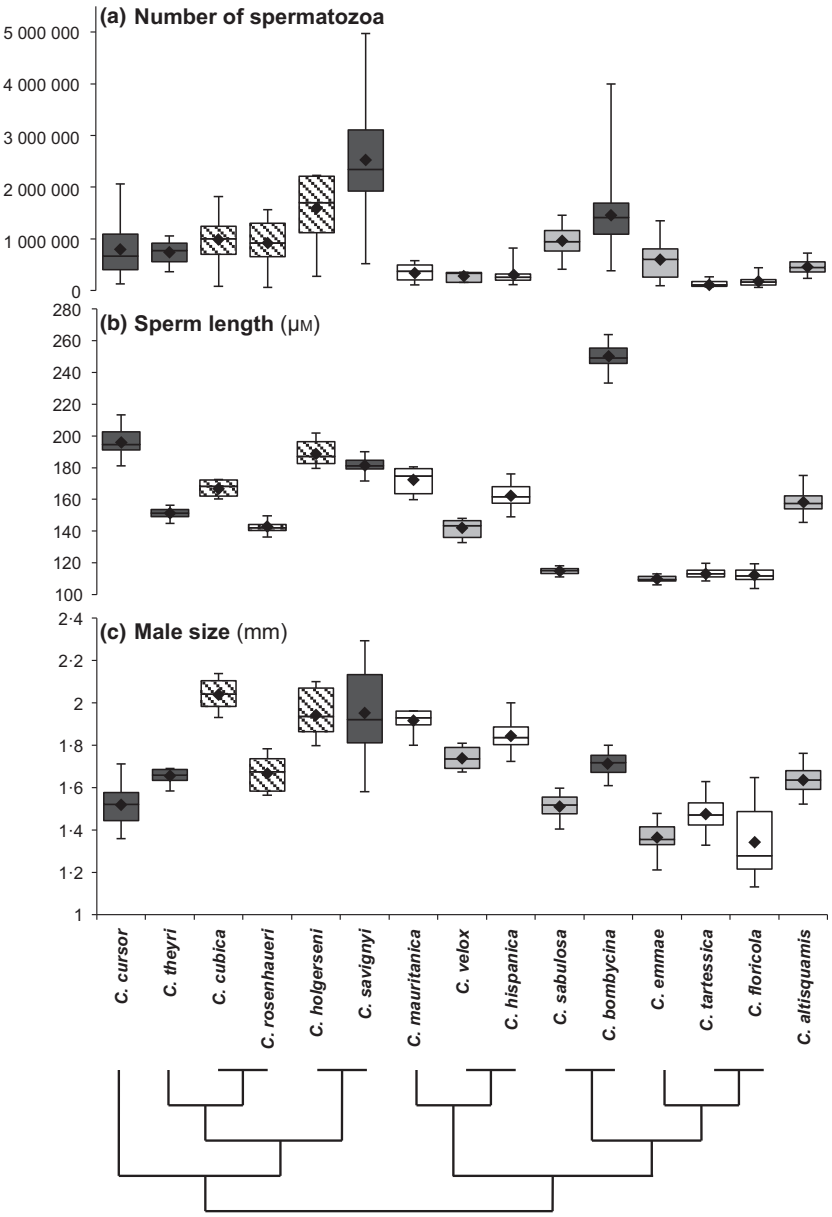

Fig. 1. Sperm production given as the number of spermatozoa stored in the accessory testes (a), sperm length (b) and male size (c) of different species of *Cataglyphis* ants, shown as box-and-whiskers plots. For each species, the overall mean estimate is marked as a diamond and the median as a horizontal line. The lower and upper ends of each box represent the 25% and 75% quartiles of the data, respectively, and the whiskers show the entire range of the data. Dark grey boxes correspond to obligatory multiple mated species (*C. bombycina*, *C. cursor*, *C. savignyi* and *C. theyri*), medium grey boxes represent multiple–single mated species (*C. altisquamis*, *C. emmae*, *C. sabulosa* and *C. velox*), and unfilled boxes indicates single–double mated species (*C. hispanica*, *C. floricola*, *C. mauritanica* and *C. tartessica*). Boxes with diagonally striped bars correspond to species for which the mating system class is unknown (*C. cubica*, *C. holgerseni* and *C. rosenhaueri*). Data are given in a simplified phylogeny for the species included in the study, based on the following four gene regions: nDNA *Abdominal-A*, nDNA *Wingless*, nDNA *Long-Wavelength Rhodopsin* (LR) and mtDNA *Cytochrome Oxidase I* (no molecular data are available to infer the phylogenetic position of the species *C. altisquamis*).

reduction from multiple to single mating influences male ejaculate characteristics.

We investigated the relationship between sperm production, sperm length, sperm length variation, male size and levels of sperm competition, in a comparative study of 15 *Cataglyphis* species using phylogenetically controlled analyses. We estimated the level of sperm competition from paternity frequency, here defined as the number of males that father the offspring of a single queen (Boomsma & Ratnieks 1996). Sperm production was measured directly by quantifying the number of spermatozoa produced by males, rather than indirectly by using proxy measures of production such as accessory testes size. We first examined whether sperm production and sperm length are related to sperm competition intensity, by comparing both sperm traits between different mating system classes that differ in the level of polyandry. Secondly, we studied whether sperm characteristics are associated with the level of sperm competition inferred from paternity frequency. Given that sperm traits (size and number) may be correlated with male size (Birkhead & Møller 1998), we also included this variable as a predictor. Finally, we analysed whether variations in male size or sperm traits are associated with the levels of sperm competition across species.

Materials and methods

SPECIMENS AND SPERM COLLECTION

All *Cataglyphis* species show very similar ecology: they are highly thermophilic, and inhabit arid lands and desert (Lenoir *et al.* 2009). Our data set contained 15 species of *Cataglyphis* ants. For each species, sperm number, sperm length and paternity frequency data (when available) originated from the same study population. This requirement is important since biogeographic variations in

population-specific paternity frequencies were previously documented in ants (e.g. Van der Have, Boomsma & Menken 1988; Boomsma & Van Der Have 1998; Schlüns *et al.* 2009; Corley & Fjerdingstad 2011; Suni & Eldakar 2011). In total, we gathered information on sperm traits from a sample of 308 males across 15 species (see Table S1 in Supporting information).

Colonies of *Cataglyphis* were sampled during the period of sexual production, between 2009 and 2012 in Spain (*C. floricola, C. hispanica, C. rosenhaueri, C. tartessica, C. velox*), Morocco (*C. bombycina, C. cubica, C. emmae, C. mauritanica, C. theyri*), Israel (*C. altisquamis, C. holgerseni, C. sabulosa, C. savignyi*) and France (*C. cursor*). Males were sampled directly during excavation, or after colonies were transported to the laboratory where males emerged. Males were decapited and then dissected under a Leica MZ6 stereomicroscope (Leica Microsystems, Wetzlar, Germany) in Ringer's solution (NaCl 0·15 M, $CaCl_2$ 0·004 M, KCl 0·002 M, $NaHCO_3$ 0·024 M). The heads were stored separately at −4 °C for subsequent size analyses (see below). All the males sampled had degenerated testes, and their spermatozoa were stored in the accessory testes, ready to be ejaculated, indicating that they were fully mature. For each male, both accessory testes were transferred to a microscope slide in 200 μL Ringer's solution. The accessory testes were punctured and carefully squeezed; the outflowing semen was transferred into a vial, and Ringer's solution was added to bring the final volume of the stock solution of semen to 500 μL.

SPERM PRODUCTION

We estimated sperm production by determining the actual number of spermatozoa stored in males' accessory testes. Spermatogenesis in ants is discontinued in adult life; males reach adulthood with a fixed amount of spermatozoa stored in the accessory testes and are not able to replenish sperm throughout their adult life (Hölldobler & Wilson 1990). Thus, the number of sperm stored in the accessory testes provides a complete measure of total investment into sperm production.

The number of spermatozoa produced by each male was determined by flow cytometry analysis, according to the experimental protocol of Cournault & Aron (2008). The stock solution of semen was gently vortexed for 30 s, and five aliquots of 20 μL were immediately taken, placed in test tubes, and their final volume brought to 1 mL with a DAPI-staining solution (4,6-diamidino-2-phenylindole; CyStain UV Ploidy, PARTEC©, Münster, Germany). The tubes were vortexed for 15 sec, and the concentration of sperm cells in each was estimated by using a PA-I flow cytometer (PARTEC©). Data were collected for a volume of 200 μL from each test tube, at a speed of 1–5 μL/s with a pre-run time set to 3 (as recommended by the manufacturer). Determination of the number of spermatozoa was performed by gated counts, *that is* number of fluorescent events measured and displayed in the corresponding histogram section. By using this method, we obtained five measures of the number of spermatozoa in 200 μL for each male, each measure corresponding to 1/125 of the accessory testes. The coefficient of variation among the five aliquots per male was reasonably low (mean ± SD = 13 ± 7%; min-max: 4–26%), indicating weak differences between sperm samples. We estimated the number of spermatozoa produced by each male by multiplying the number of sperm cells in every 200 μL sample by 125, and calculating the mean of five counts. Mean sperm production values for each species were calculated from the means of each male of that species.

SPERM LENGTH

To measure sperm length, an aliquot of 200 μL was taken from the stock solution of semen from each male, placed in a vial and incubated with 200 μL of DAPI-staining solution ($NaH_2PO_4.H_2O$ 40 mM, $Na_2HPO_4.7H_2O$ 160 mM, pH 7·4) for 5 min. A drop of sperm/DAPI solution was smeared on a microscope slide and air-dried. The slide was then fixed with paraformaldehyde 4% (Sigma-Aldrich, Steinheim, Germany) and rinsed twice in phosphate-buffered saline (PBS) solution for 5 min. The DAPI-staining solution spreads to the end of the flagellum, allowing a very accurate measurement of the total sperm length. Photographs of spermatozoa were taken with a digital camera mounted on a Nikon Eclipse 50i microscope (Nikon Instruments, Badhoevedorp, The Netherlands) equipped with an epi-fluorescence illuminator, at 40× magnification. From these pictures, we measured the total length (to the nearest 0·01 μm) of 25 intact spermatozoa per male using IMAGEJ software (version 1.8; NIH, Bethesda, MD, USAhttp://imagej.nih.gov/ij/). We focused our analyses on total sperm length, because the sperm morphology of the species used in our analyses does not allow a clear distinction between different sperm components (head, midpiece and flagellum). Mean sperm length values for each species were calculated from the means of the lengths of 25 spermatozoa per male of that species.

PATERNITY FREQUENCIES

Paternity frequency (i.e. the number of males that contribute to offspring production of a queen), instead of queen mating frequency (i.e. the actual number of males a queen mates with), was used as an estimate of sperm competition level. The reason being that, in social insects, mating frequency is difficult to determine because mating typically occurs during large nuptial flights tens of meters above ground. Moreover, queens only rarely mate under laboratory conditions. Both estimates may be quite different due to whether or not sperm are transferred to the queen spermatheca and used to fertilize the eggs. Therefore, measuring paternity frequency is the best way to estimate the number of matings per queen in social Hymenoptera (Baer 2011; Jaffé *et al.* 2012).

Data on paternity frequencies were collected from the literature (see Table S2); they are based on the number of patrilines found from mother–offspring genetic combinations. All paternity studies were considered methodologically robust with respect to analyses techniques (microsatellites). No species was singly mated (strict monandry); this is consistent with studies in other ants showing that species descending from ancestors with obligate multiple mating never return to complete single mating (Sumner *et al.* 2004; Kronauer & Boomsma 2007). According to Boomsma & Ratnieks (1996) classification, species were grouped as follows: obligatory multiply mated (paternity frequency always ≥ 2 and often ≥ 4 mating per queen), multiple–single mated (paternity frequency usually > 1, with a minority of queens singly mated) and single–double mated (paternity frequency usually = 1, with a minority of queens doubly mated) (see Results).

MALE SIZE

We measured seven morphological traits on a subsample of males from three species (*Cataglyphis sabulosa*, $n = 21$; *C. rosenhaueri*, $n = 20$; *C. bombycina*, $n = 20$): head width (eyes included), head width between eyes, scape length, thorax length, tibia and femur lengths of the hindleg, and pronotum width. Body parts were photographed using light microscopy at 50× magnification (MZ6 stereomicroscope; Leica Microsystems, Wetzlar, Germany) and measured to the nearest 0·01 mm using IMAGEJ. Maximum head width (eyes included) was highly correlated with all other body measurements (multiple regression coefficients: all $r > 0{\cdot}94$; ANOVAS of multiple regressions: all $P < 0{\cdot}002$) and reproducible with a low standard error of mean (sem < 3·2%). Therefore, maximum head width was used as a proxy for male body size for all species sampled. The identity of the males and their sperm counts (see below) were unknown to the experimenter doing the morphometry.

STATISTICAL ANALYSIS

All analyses were performed on the 15 species of *Cataglyphis*, except the analysis including paternity frequency where data for three species (*C. cubica*, *C. rosenhaueri and C. holgerseni*) were lacking. Prior to analyses, all continuous variables were checked for normality using a Shapiro–Wilk test. Variables were log10-transformed to achieve normal distribution and because allometric relationships may occur between them (Baer *et al.* 2009). As a standardized measure of variation, we used the sample-size-corrected coefficient of variation (CV* = (SD/mean × 100) × (1+(1/4N))) (Sokal & Rohlf 1995), denoted as CV_{bm} for the between-male CV* in male size, sperm production and mean sperm length, and CV_{wm} for the mean within-male CV* in sperm length.

We tested whether the number of collected males allowed for a robust estimate of the within-species variance through a modified rarefaction analysis. We used the functions *sample* and *var* in R version 3.0.1 (R Development Core Team 2013) to estimate the variance of subsamplings of a fixed number of males (500 replicates), and plotted variance as a function of the number of males in the subsamplings. Simulations showed that a minimum of 10 males gives a reasonable estimate of the within-species variance for sperm production, sperm length and male size. For the two species with the lowest sample numbers (*C. holgerseni* and *C. mauritanica*), variance of male size remained dependent of the subsamplings and our estimation of variance was therefore less robust.

To analyse the interaction between levels of sperm competition, sperm traits and male size, a series of statistical tests were performed using Statistical Package for the Social Sciences (SPSS v.20; IBM Corporation, Armonk, NY, USA). Species may share character values as a result of a common ancestry rather than independent evolution (Harvey & Pagel 1991) meaning species data may not be fully independent (Felsenstein 1985). We therefore performed phylogenetically controlled generalized least-squared (PGLS) regressions (Pagel 1999; Freckleton, Harvey & Pagel 2002). PGLS regression uses maximum likelihood ratio tests to estimate a phylogenetic association parameter λ, which assesses the degree of phylogenetic dependence in the data. If λ values are close to 0, the variables are likely to have evolved independently of phylogeny. Alternatively, λ values close to 1 indicate strong phylogenetic association of the variables, and phylogenetic corrections are necessary. The maximum likelihood value of λ was compared with models with fixed λ = 1 and λ = 0 by means of a log-likelihood ratio test. The estimation of λ values and GLS analyses were performed using the statistical package *caper* (Orme 2013), which requires the *ape* package (Paradis, Claude & Strimmer 2004) along with the packages *mvtnorm* (Genz & Bretz 2014) and *MASS* (Venables & Ripley 2002), in R version 3.0.1.

We constructed a phylogenetic topology of the 15 species based on a final fragment of four gene regions (nDNA *Abdominal-A*, nDNA *Wingless*, nDNA *Long-Wavelength Rhodopsin* and mtDNA *Cytochrome Oxidase I*; L. Leniaud, personal communication) using programs freely available from the phylogeny.fr Web server (Dereeper *et al.* 2008) (MUSCLE for multiple alignment, Edgar 2004; GBLOCKS for alignment curation, Castresana 2000; PHYML for phylogeny, Guindon *et al.* 2010; and TREEDYN for tree drawing, Chevenet *et al.* 2006).

Results

Sperm were collected from 308 males originating from 73 colonies and 15 *Cataglyphis* species (mean number of males per species ± SE = 20·53 ± 3·34, range: 7–54; mean number of males per colony ± SE = 4·22 ± 0·29, range: 1–10; mean number of colonies per species ± SE = 4·87 ± 0·65, range: 1–10).

Sperm production, sperm length and male size varied considerably across species (Fig. 1a–c; see Table S1). The number of spermatozoa produced by males ranged from 128 800 to more than 2·5 millions (CV = 92·48%), mean sperm length varied more than twofold across species, from 109 to 250 μm (CV = 26·13%), and mean head size varied from 1·34 to 2·04 mm (CV = 14·49%). Analyses of variance (ANOVA) on sperm number, sperm length and male size showed that differences between species accounted for, respectively, 75%, 98% and 81% of the variance components, whereas variation between males within species explained 25%, 2% and 19% of the variance. For sperm length, hierarchical (nested) ANOVA indicated that about 96% of the total variation in sperm length was explained by the interspecific variation, whereas intraspecific and intramale variations contributed only around 1% and 3%, respectively.

Paternity frequency data (see Table S2) fit the categorization into three classes of mating systems: obligatory multiple mating, multiple–single mating and double–single mating. Data on sperm production, sperm length and male size are given for each species and by mating class, in Figs 1 and 2, respectively. The four species *C. cursor, C. theryi, C. savignyi* and *C. bombycina* belong to the obligatory multiple mated class. They have both substantially elevated sperm numbers and sperm lengths, suggesting obligate multiple mating selects for numerous and long sperm cells. *C. velox, C. sabulosa, C. emmae* and *C. altisquamis* fit with the multiple–single mating class. All show a reduction in sperm number and sperm length relative to obligate multiple mated species. However, as shown in Fig. 1, males of *C. sabulosa* and *C. emmae* maintain elevated sperm production but minimal sperm length, whereas *C. velox* and *C. altisquamis* have reduced sperm production but elevated sperm length. Finally, four species match the single–double mating system class: the two sister species *C. tartessica* and *C. floricola*, and the closely related *C. mauritanica* and *C. hispanica*. They all have minimal sperm numbers. However, these species show substantial sperm length variations: *C. mauritanica* and *C. hispanica* have elevated sperm lengths, while the two species *C. tartessica* and *C. floricola* have minimal sperm length (Fig. 1). Regarding male size, males from the obligatory multiple mated class are slightly larger than those from the two other categories; however, large variations occur in male size within each mating system. These results show that sperm production and sperm length vary with the mating system class, suggesting that both matter in *Cataglyphis*.

Our simplified phylogeny given in Fig. 1 reveals two major clades. A first clade on the left side of the figure with six species: three obligatory multiple mated species that are not monophyletic (*C. cursor*, *C. theryi* and *C. savignyi*) and three species for which no data on the mating system is available (*C. cubica*, *C. rosenhaueri* and *C. holgerseni*). These six species are characterized by elevated sperm numbers and relatively elongated sperm cells. This

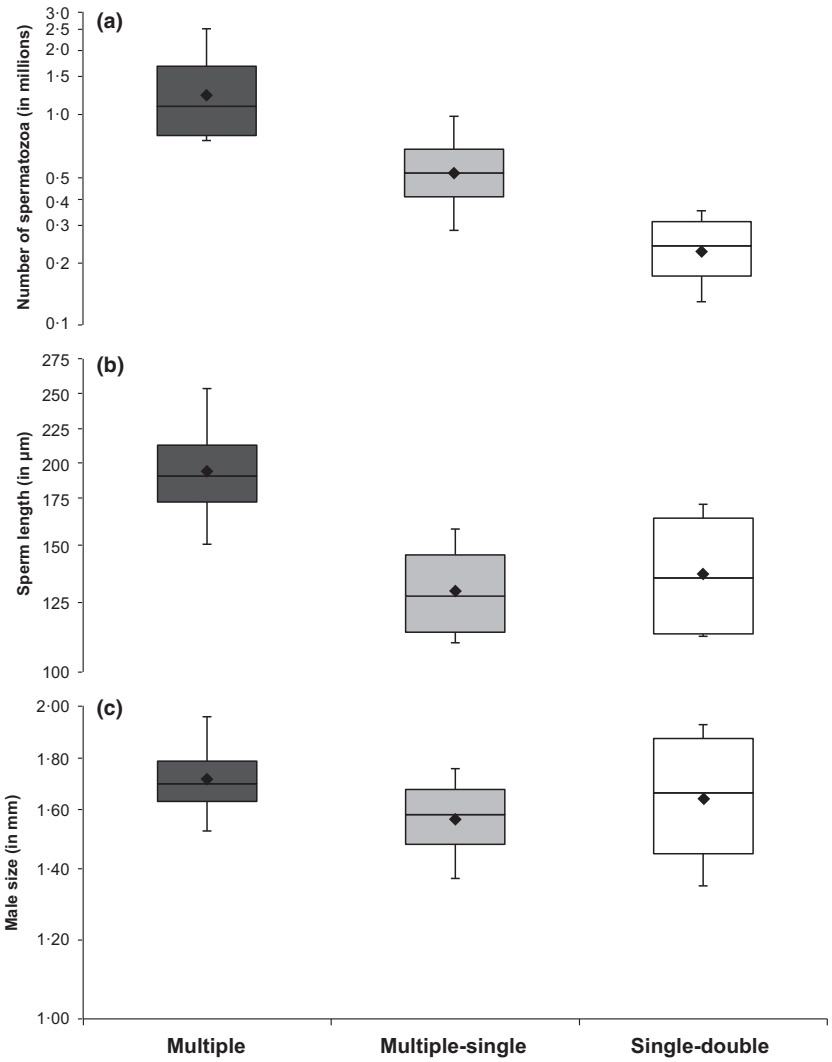

Fig. 2. Number of spermatozoa produced per male (a), sperm length (b) and male size (c) in obligatory multiple mated species ($n = 4$), multiple–single mated species ($n = 4$) and single–double mated species ($n = 4$) of *Cataglyphis* ants. Mean estimate is marked as a diamond and the median as a horizontal line. The lower and upper ends of each box represent the 25% and 75% quartiles of the data, and the whiskers show the entire range of the data. Difference after phylogenetic control using GLS between the three mating system classes is statistically significant for sperm production only ($P = 0{\cdot}008$, $F = 8{\cdot}33$). The *y*-axis is log10-transformed; labels on the *y*-axis correspond to the original untransformed values.

similarity of sperm traits and their phylogenetic relationship suggest that they all belong to the same mating system class. The second major clade is represented by the other species. These are characterized by a reduction in paternity frequency. All species belonging to this second major clade, except *C. bombycina*, have intermediate to reduced values of sperm number and sperm length. The silver ant *C. bombycina* contrasts with the other members of the clade, because it belongs to the obligatory multiple mated class and shows large number of sperm and the longest spermatozoa. Overall, these trends indicate some phylogenetic inertia in the relationship between sperm traits and sperm competition level.

Phylogenetically controlled multiple regression analyses confirmed the negative association between the reduction in the level of sperm competition and the number of spermatozoa produced per male, with males of obligatory multiple mated species showing a significantly higher investment in sperm production than males from multiple–single and single–double mated species (slope ± SE = 2·64 ± 1·98, $P = 0{\cdot}009$) (Fig. 2a; see Table S3). Furthermore, the number of spermatozoa produced per male decreased significantly with reduction in paternity frequency (slope ± SE = 0·70 ± 0·13, $P = 0{\cdot}001$) (Fig. 3a; see Table S3). This trend was also clearly negative within the obligatory multiple mated and the multiple–single mated classes, but not in the single–double mated one (Fig. 3a). Thus, as multiple mating gets reduced, sperm production is lowered. In contrast, neither sperm length nor male size was significantly associated with the mating system classes (sperm length: $P = 0{\cdot}223$; male size: $P = 0{\cdot}207$) (Fig. 2b,c; see Table S3) or the number of patrilines (sperm length: $P = 0{\cdot}355$; male size: $P = 0{\cdot}140$) (Fig. 3b,c; see Table S3). As judged from the values of λ (all < 0·001; Table S3), these associations were not influenced by phylogeny. Finally, PGLS analyses indicated no association between sperm number and male size (slope ± SE = 0·05 ± 0·04, $P = 0{\cdot}276$) or between sperm number and sperm length (slope ± SE = 1·35 ± 0·75, $P = 0{\cdot}095$). Sperm length was, however, positively associated with male size (slope ± SE = 0·29 ± 0·10, $P = 0{\cdot}014$). Altogether, these results show that investment in sperm production decreases with decreasing sperm competition in *Cataglyphis* desert ants.

Across species, we found no evidence that variation in male size or sperm traits (production or size) was associated with the levels of sperm competition. Coefficients of variation of sperm count, sperm length (within-male and between-male variance within species) or male size were associated neither with the mating system nor with the paternity frequency (PGLS regression analyses with the mating system as dependent variable: $r^2 = 0{\cdot}18$, all $P > 0{\cdot}383$; with the paternity frequency as dependent variable: $r^2 = 0{\cdot}23$, all $P > 0{\cdot}429$; see Table S4).

Discussion

Previous studies have shown that obligate multiple mating is the ancestral state in *Cataglyphis* desert ants and that reduction in queen mating frequency evolved secondarily in some clades (Leniaud, Darras & Aron 2010; S. Aron, L. Leniaud, P. Mardulyn, in preparation). Reversal was, however, not complete since no species was found to be exclusively singly mated. This pattern is consistent with other studies in ants showing that reduction in mating frequency does not produce absolute reversal to exclusive single mating (Boomsma, Kronauer & Pedersen 2009). Here, paternity frequency data allowed us to identify three mating system classes: obligatory multiple mated, multiple–single mated and double–single mated.

SPERM PRODUCTION DECREASES WITH REDUCTION IN THE LEVEL OF SPERM COMPETITION

Our findings show that paternity frequency drives the evolution of male investment in sperm production in *Cataglyphis* ants. Across the species sampled, and after controlling for phylogeny, a decrease in both the 'risk' of sperm competition (obligatory multiple mated > multiple–single mating > single–double mating) and the 'intensity' of sperm competition (the actual paternity frequency) selects for smaller ejaculates. To the best of our knowledge, this is

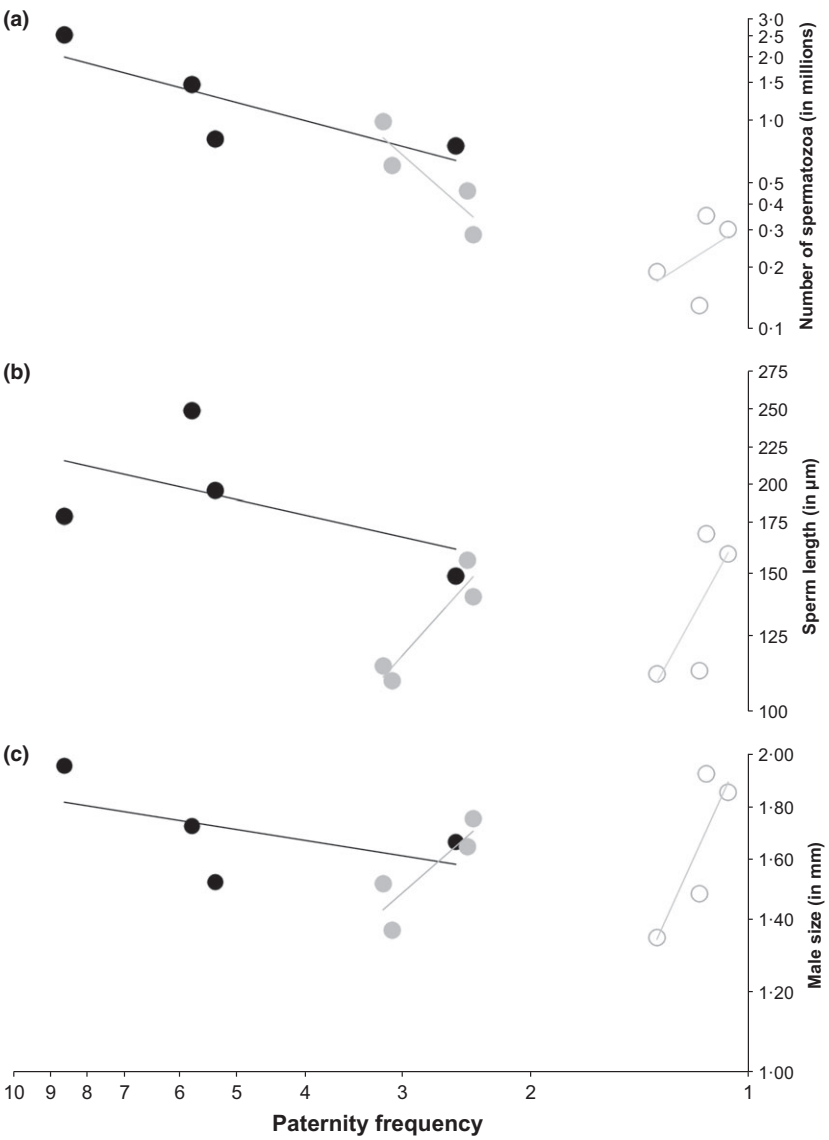

Fig. 3. Relation between mean paternity frequency (i.e. the mean number of males that father offspring of a queen) and the number of spermatozoa produced per male (a), sperm length (b) and male size (c) in 12 species of *Cataglyphis* ants. Dark grey dots correspond to obligatory multiple mated species, medium grey dots to multiple–single mated species, and unfilled dots to single–double mated species. The x- and y-axes are log10-transformed; labels on both axes are untransformed values.

the first time that reduction in sperm competition due to reversal from multiple to single mating is shown to influence sperm traits in an organism. Our comparative results suggest that increased production of sperm provides a fitness benefit to males under conditions of sperm competition in eusocial Hymenoptera. In line with this, previous experimental studies showed that male paternity share is related to the number of sperm cells transferred to the female storage organ in the honeybee *Apis mellifera* (Schlüns *et al*. 2003) and in leaf-cutting ants (*Atta colombica*, Holman *et al*. 2011; *Acromyrmex echinatior*, Stürup *et al*. 2014). In social insects, a single study investigated the effect of the intensity of sperm competition on investment in sperm production. Baer & Boomsma (2004) reported that the relative size of the accessory testes was positively correlated with the paternity frequency in male fungus-growing ants. Our results showing that sperm production decreases with reduction in paternity frequency across species are consistent with this observation, and they provide the first direct evidence that the *actual* number of spermatozoa produced (rather than testes size) varies according to the detected number of matings per queen.

Two competing factors may potentially explain the differences in the number of sperm cells produced by males found in this study: male size and colony size. (i) Male size may ultimately determine the amount of sperm that can be produced. Sperm production is indeed positively correlated with body size in bees (Garofalo, Zucchi & Muccillo 1986; Schlüns *et al*. 2003) and ants (Wiernasz *et al*. 2001). In *Pogonomyrmex* harvester ants, females are highly polyandrous and, during copulation, larger males transfer a greater proportion of their sperm than smaller males, and individual fitness is predicted to increase as a function of male size (Wiernasz *et al*. 2001). We found no correlation between male body size and sperm count across species, suggesting that male body size is not a strong predictor of sperm production in *Cataglyphis* desert ants. Additionally, male size was influenced neither by the mating system (obligatory multiple mating, multiple–single mating, single–double mating) nor by the paternity frequency. (ii) Colony size is also expected to exert strong selection on sperm production in social insects, because large mature colonies are ultimately limited by the amount of sperm stored by queens at the time of mating. Thus, across species, sperm number is predicted to be augmented with increasing colony size to ensure storage of a greater number of spermatozoa in the female sperm storage organ (Boomsma, Baer & Heinze 2005; Baer *et al*. 2009). This hypothesis seems unlikely to account for the variation in sperm production across species found in our study. In *Cataglyphis* ants, the size of mature colonies is relatively small, ranging from 180 to approximately 3000 workers (estimates obtained from the literature and our own field notes; see Table S5). The average number of spermatozoa produced across the 15 species studied is not associated with mature colony size (PGLS regression analysis: slope $\pm$ SE = 0·34 $\pm$ 0·20, $P = 0{\cdot}104$, $r^2 = 0{\cdot}21$). Altogether, this supports the view that constraints on male and colony size have little direct influence, if any, on sperm production and that sperm competition is the primary factor shaping ejaculate size in *Cataglyphis* ants.

SPERM LENGTH AND MALE SIZE RESPOND WEAKLY TO SPERM COMPETITION LEVEL

Our data show that sperm length and male size respond much less than ejaculate size to reduction in sperm competition intensity. These variables are associated neither with the mating system classes nor with paternity frequency, suggesting that sperm competition has not been a major selective force for sperm length or male size evolution in *Cataglyphis*. Similar results were obtained in the fungus-growing ants, where mate number affects sperm length to a minor extent (Baer *et al*. 2009). Although not significant when controlling for phylogeny, our results still indicate a reduction in sperm size, with males from the obligatory multiple mated class having longer sperm than males belonging to the multiple–single and single–double classes (Fig. 2b); sperm length also varies slightly with decreasing paternity frequency (Fig. 3b). Because there is no complete reversal to exclusively single mating in *Cataglyphis*, potentials for sperm competition still persist in the multiple–single and single–double classes. In other words, even if there is a premium for longer sperm having a higher likelihood of being stored (because of their swimming speed), it matters little whether ejaculates compete with one or more other ejaculates. Thus, selection on sperm length reduction is probably weak.

How sperm competition influences sperm size remains unclear and literature consistently provides little evidence for a general pattern of selection acting on sperm length in animals (Simmons & Fitzpatrick 2012; Simpson *et al*. 2014). Sperm cells are energetically costly to produce and it has long been assumed that a trade-off exists between sperm size and number (Parker 1970a). In eusocial Hymenoptera, males cease spermatogenesis after emergence and one may easily expect a trade-off between making many short spermatozoa and few longer spermatozoa. Sperm competition should select for production of longer and faster sperm. However, constraints on sperm storage may prevent such development because females have to store a lifetime supply of sperm early in life and the spermatheca has a limited capacity (Boomsma, Baer & Heinze 2005; Baer *et al*. 2009). Thus, sperm length should decrease with increasing paternity frequency and/or mature colony size because more can be stored to fulfil the lifetime reproductive potential of the queen and her mate(s). Consistent with this prediction, in the fungus-growing ants, sperm cells are longer in the basal genera with small, short-lived colonies and decrease in length with increasing colony size and queen longevity (Villesen *et al*. 2002; Baer & Boomsma 2004). We found no evidence for a trade-off between sperm length and number in *Cataglyphis* ants. At least two

explanations may account for this result. First, constraints on sperm storage could be relaxed in this genus because colonies are typically small, from a few hundreds to a few thousands individuals (see Table S5). Unfortunately, we have no reliable data on queen longevity and spermatheca size for any species of *Cataglyphis*, and precise estimates of mature colony size are available for only half the species studied. We are therefore unable to test for a possible association between sperm length and queen longevity, spermatheca size or mature colony size. Secondly, it has been suggested that sperm production constraints may directly influence sperm length (Parker & Pizzari 2010). Production constraints may stem from limitation on the resources that smaller males can accumulate during development, from smaller males having small accessory testes to store sperm, or from the need to remain sufficiently light to efficiently participate in nuptial flights. A significant relationship between sperm length and sexual dimorphism was indeed reported in the fungus-growing ants, with sperm being shorter in species with small males relative to queens (Baer *et al.* 2009). Our PGLS analyses show a positive association between sperm length and male body size across species; that is, sperm is shorter in species with small males. While this suggests that sperm production constraints may be of primary importance in determining sperm length, it may also result from allometric relationships and/or genetic effect if sperm length is heritable and correlated with genes coding for body size. Obviously, further research will be necessary to settle these issues.

VARIABILITY IN SPERM TRAITS IS NOT ASSOCIATED WITH PATERNITY FREQUENCY

Sperm competition is predicted to reduce variability in sperm traits to favour an optimal sperm phenotype enhancing a male's reproductive success (Parker 1993; Parker & Begon 1993). In agreement with this prediction, there is ample correlational evidence that variation in sperm traits is negatively associated with the level of post-copulatory sexual selection in a variety of species (Calhim, Immler & Birkhead 2007; Immler, Calhim & Birkhead 2008; Kleven *et al.* 2008), including social insects (Fitzpatrick & Baer 2011). The latter was a comparative study across 27 species of eusocial bees and ants, and showed an inverse relationship between paternity frequencies and coefficients of both between-male (CV_{bm}) and within-male (CV_{wm}) variation in sperm length. Our results contrast with these findings: variations in sperm number and sperm length were found to be associated neither with sperm competition risk nor with sperm competition intensity. Two explanations may account for the lack of canalization of sperm length with increased levels of sperm competition in *Cataglyphis*: a weak selection on sperm length, or a stabilizing selection favouring an optimal sperm length for each species that is independent of the mating system. Our data support the first hypothesis. First, we found no response of sperm length to varying levels of sperm competition; secondly, there are large differences in sperm length variation across species (see Table S1).

The observation of lower sperm production when competition is reduced suggests a cost for producing more sperm balanced by the benefit of a large sperm count under polyandry. One would therefore expect the variability in the number of sperm produced to be negatively associated with increased paternity frequency. Yet, such a trend in sperm number variation was not apparent in our study; our data reveal a considerable interindividual variation in sperm production in *Cataglyphis* (Fig. 1). One explanation for the apparent lack of selection on sperm number variation is that variance in sperm production is generated by environmental factors such as food availability, temperature or immunological challenges. In Hymenoptera, spermatogenesis occurs during the pupal stage and is usually completed at the emergence of the imago. Conditions under which males are reared may therefore be critical since they determine the resources available for gamete production. Gametic investment was indeed shown to be resource-dependent in the stingless bee *Melipona beecheii* (Pech-May *et al.* 2012) and the honeybee *Apis mellifera* (Czekonska, Chuda-Mickiewicz & Samborski 2014), where males reared under reduced pollen reserves produce significantly lower numbers of spermatozoa than those reared under supplemented reserves. In contrast, dietary restrictions after hatching do not affect male fecundity in the honeybee (Stürup *et al.* 2013). Sperm competition game models assume that males mate multiple times, that they can differentially invest in the number of sperm transferred per ejaculate, and that there is a trade-off between ejaculate expenditure and acquisition of new mates (i.e. greater sperm expenditure may increase success in sperm competition, but reduces the total number of mating by causing males to spend more time out of mate-searching to replenish sperm supplies) (Parker 1970b, 1982; Parker & Pizzari 2010). These models cannot be applied as such to eusocial Hymenoptera, where males emerge with a finite amount of sperm, then take part in energetically expensive nuptial flights to find a mate, and typically gain a single mating before they die. In this situation, it is the resource allocation to gametes vs. that to the soma that is under selection, rather than (in species with gamete replenishment) the sperm number per ejaculate. In other words, the trade-off lies between a male's ability to store sufficient energy to sustain an expensive mating flight and his success in gaining fertilizations afterwards (i.e. the number of sperm produced). In support of this, a negative correlation between thorax weight and sperm content was documented in males of the leafcutter ant *Atta colombica* (Fjerdingstad & Boomsma 1997). Males having a larger thorax (and longer wings) produce less sperm, but are probably better fliers, which increases their chance of obtaining matings. We predict that males of eusocial Hymenoptera should invest in somatic tissue to pass the threshold needed to undertake their single reproductive event, and then invest the remaining resources in sperm production.

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Data accessibility

Data are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.v7000 (Aron *et al*. 2015).

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Supporting Information

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

Table S1. Male size, sperm number, sperm length and sperm length variation in *Cataglyphis*.
Table S2. Paternity frequency values and mating system classes in *Cataglyphis*.
Table S3. PGLS analyses of male size, sperm number and sperm length in relation to the mating system class and paternity frequency.
Table S4. PGLS analyses of variance in male size, sperm number and sperm length in relation to the mating system class and paternity frequency.
Table S5. Mature colony size of *Cataglyphis* species.