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Random sperm use and genetic effects on worker caste fate in *Atta colombica* leaf-cutting ants

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Abstract

Sperm competition can produce fascinating adaptations with far-reaching evolutionary consequences. Social taxa make particularly interesting models, because the outcome of sexual selection determines the genetic composition of groups, with attendant sociobiological consequences. Here, we use molecular tools to uncover some of the mechanisms and consequences of sperm competition in the leaf-cutting ant *Atta colombica*, a species with extreme worker size polymorphism. Competitive PCR allowed quantification of the relative numbers of sperm stored by queens from different males, and offspring genotyping revealed how sperm number translated into paternity of eggs and adult workers. We demonstrate that fertilization success is directly related to sperm numbers, that stored sperm are well-mixed and that egg paternity is constant over time. Moreover, worker size was found to have a considerable genetic component, despite expectations that genetic effects on caste fate should be minor in species with a low degree of polyandry. Our data suggest that sexual conflict over paternity is largely resolved by the lifetime commitment between mates generated by long-term sperm storage, and show that genetic variation for caste can persist in societies with comparatively high relatedness.

Keywords: competitive PCR, cryptic female choice, fair raffle, genetic caste determination, sperm competition

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Introduction

Sperm competition (Parker 1970) is a widespread phenomenon with manifold evolutionary and ecological consequences (e.g. Miller & Pitnick 2002; Boomsma *et al.* 2005; Holman & Snook 2008; Price *et al.* 2008; den Boer *et al.* 2010; Manier *et al.* 2010). Sperm competition occurs whenever ejaculates of different males compete for a common set of eggs, and selects for traits that afford males a greater share of paternity in polyandrous females. Males frequently produce more sperm to numerically out-compete those of rivals, and may possess adaptations that reduce the number of competing rival sperm (Birkhead & Møller 1998; Simmons 2001; den Boer *et al.* 2010; Manier *et al.* 2010). 'Fair raffle' sperm competition is a form of null model in which the fertilization success of each competing male is equal to the proportion of sperm belonging to that male in the fertilization set (e.g. Parker 1990). Deviations from the fair raffle show that some males' sperm have more chance of achieving fertilization than those of other males, for example because they differ in traits that affect fertilization success (e.g. insemination order, motility or survival; Snook 2005), or because females bias paternity towards particular males ('cryptic female choice'; Eberhard 1996).

The eusocial Hymenoptera are unique among insects in many respects, including their mating biology. Reproductive activity is typically concentrated into a single brief 'mating flight', after which queens store sperm for unusually long periods (up to 20–30 years) and never mate again (Boomsma *et al.* 2005). This mating system imposes unique selective pressures on males, e.g. to produce sperm that remain viable

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and competitive for many years. Additionally, hymenopteran males typically only receive fitness returns from daughters that develop into queens, which tend to be produced months or years after the mating flight. This mating system reduces interlocus sexual conflict, because a male's fitness depends on the long-term survival of his mate and her colony, constraining the evolution of competitive male traits that reduce the survival of either (Boomsma *et al.* 2005). Males are also expected to have comparatively little power to influence paternity after the mating flight, because the majority of fertilizations occur long after the male has died and the nonsperm components of the ejaculate (e.g. seminal proteins; Baer *et al.* 2009) have broken down (den Boer *et al.* 2010).

Despite these evolutionary constraints, specialized male adaptations to sperm competition have been reported, including chemically complex seminal fluid (Baer *et al.* 2009) that lowers the survival of rival male sperm while preserving self-sperm (den Boer *et al.* 2010; King *et al.* 2011). Other putative male adaptations to sexual selection include mating plugs (Baer *et al.* 2000; Mikheyev 2003; Baer 2011) and detachable male genitalia (Wilhelm *et al.* 2011). Large variation in paternity skew (the difference in proportion of offspring fathered by each male; Boomsma & Ratnieks 1996) has been reported both within and across species (e.g. Boomsma & Sundstrom 1998; Boomsma & Van der Have 1998), although the proximate and ultimate causes of this variation remain virtually unknown. The intracellular bacterium *Wolbachia*, which can influence paternity skew by rendering some mating types infertile (cytoplasmic incompatibility; Werren 1997), is also present in many social Hymenoptera (e.g. Van Borm *et al.* 2001), but the consequences of infection are largely unknown.

Sperm competition in social insects is of particular interest because of the sociobiological consequences of mating systems. The degree of polyandry and the outcome of postcopulatory sexual selection together determine relatedness among the queen's offspring. Relatedness is a key parameter in the origin and maintenance of eusociality, because altruistic traits such as worker sterility are only selectively favoured when they provide enough benefit to sufficiently close relatives (Hamilton 1964; Boomsma 2007, 2009). Within-colony relatedness also affects the degree of queen-worker conflict over male production and the sex ratio (Sundström & Boomsma 2000), as well as the degree of conflict between worker full-sibling lineages (patrilines) over the paternity of new queens (Wenseleers 2007). Moreover, genetic factors are now known to influence the development of females into queens or different worker morphs (reviewed in Anderson *et al.* 2008; Schwander *et al.* 2010). Polyandry has been suggested to select for greater genetic variation in caste fate, because only genetically diverse colonies can contain multiple genotypes with different biases towards particular female morphs. Contrary to this intuitive expectation, the degree of genetic caste determination across species is not clearly correlated with polyandry, suggesting a more complex picture (Schwander *et al.* 2005, 2010). A complete understanding of the origin and maintenance of eusociality and genetic caste determination therefore requires an appreciation of reproductive biology, including sperm competition when queens mate multiply.

Here, we investigate sperm use and genetic caste determination in the leaf-cutting ant *Atta colombica*. Colonies of this species contain a single queen and up to a million workers (Hölldobler & Wilson 1990), which are classified into three nondiscrete size classes termed minors, media and majors (~2–15 mm in length). Smaller workers preferentially tend the brood and fungus garden, while larger workers specialize in foraging and colony defence (Wilson 1980). Queens store sperm from a small number of males (typically 2–3; Fjerdingstad & Boomsma 1998; Baer & Boomsma 2006; Helmkampf *et al.* 2008) during a single mating flight, and males produce seminal fluid that increases the survival of their own sperm while hindering that of rivals' sperm (den Boer *et al.* 2010). However, antagonistic interactions among ejaculates are apparently halted after storage when male seminal fluid is replaced by secretions from the queen's spermatheca (the sperm storage organ) (den Boer *et al.* 2009, 2010), suggesting that overt forms of sperm competition do not continue after permanent storage. Sperm may be released from the spermatheca at random, which would lead to fair raffle competition, or there could be a bias in sperm use towards particular males. Such a bias could be caused by differences in the fertilization ability of sperm from different ejaculates, cryptic female choice, physical stratification of stored sperm leading to temporal variation in sperm use ('sperm clumping'; Boomsma & Ratnieks 1996; Wiernasz & Cole 2010) or incompatibility between male and female nuclear or cytoplasmic genotypes (*Wolbachia* has been reported to infect this species, although it is unknown whether it affects compatibility; Van Borm *et al.* 2001). Moreover, evidence for genetic effects on worker caste fate has been found in the sister genus *Acromyrmex* (Hughes *et al.* 2003; Hughes & Boomsma 2008), although its presence in *Atta* is unknown. All else being equal, we would expect genetic variation for caste to be substantially reduced in *Atta* because queen mating frequency is lower than in *Acromyrmex* (10.6 ± 1; Sumner *et al.* 2004), suggesting that genes that cause a strong predisposition towards particular worker castes would be selected against (Anderson *et al.* 2008; Schwander *et al.* 2010).

To examine how the sperm of different males are used in fertilization, we employed the under-utilized competitive polymerase chain reaction (CM-PCR) technique (see also the studies by Wooninck *et al.* 2000; Bretman *et al.* 2009; Bussière *et al.* 2010; Hall *et al.* 2010) to quantify the relative contributions of different males to the sperm stores of field-collected *A. colombica* queens. We then compared each male's share of the stored sperm to his paternity of the colony's eggs, large workers and small workers. Our results indicate that poststorage fertilization success is determined by relative sperm numbers; stored sperm are well-mixed and there are no detectable differences in sperm quality, cryptic female preferences or compatibility. We also provide compelling evidence that worker size polymorphism has a genetic component.

Methods

Ant collection and culturing

Incipient *Atta colombica* colonies ($n = 14$; 1–2 fungus chambers suggesting an age of 1–2 years) were collected from gardens and verges in Gamboa, Republic of Panama in May 2010. Whole colonies (all ants and their fungus garden) were excavated and transported to the laboratory in Copenhagen where they were kept in a climate room (~25 °C, 70% RH and a 12:12 h photocycle) and provided with bramble (*Rubus*) leaves, rice and fruit.

Egg and worker collection

The first batch of eggs was collected within 2 weeks (10 ± 2.4 days) after the colonies were excavated. Eggs were harvested by placing the queen, five workers and a small piece of brood-free fungus in a Petri dish for 24 h. The queen was then returned to her colony, while the eggs and workers were left in the Petri dish for a further 48 h to allow cell division in the eggs, increasing the amount of template DNA. All eggs were then collected with clean forceps and stored in 96% ethanol at −20 °C. We later collected 2–3 additional egg batches, the last batch being collected up to 133 days after the first (see Table S1, Supporting information).

Approximately 5 months after collection (137 ± 2 days), we freeze-killed all colonies and collected the queen and workers. The workers were sorted into small workers, defined as the smallest available minors (~3 mm long), and large workers. The large worker group contained the 20 largest workers in the colony, i.e. all available major workers (>15 mm; $n = 0$–16) plus the largest media workers (>8 mm), because young colonies typically do not produce enough majors for a full sample (mean percentage of the sample composed of majors: 47 ± 6%). All samples were stored in 96% ethanol at −20 °C.

Genotyping of eggs and adults

DNA was extracted from a random sample of 24 eggs per egg batch and 20 large and 20 small workers per colony, plus the 14 queens. Whole eggs were boiled in 100 μL 10% Chelex and 5 μL dilute (19 mg/mL) proteinase K at 95 °C for 30 min. Adult DNA (macerated whole bodies for small workers, single hind legs for large workers and queens) was extracted in 100 μL 5% Chelex. The DNA was amplified at four polymorphic microsatellite loci (Atco12, Atco13, Atco37 and Etta 5+6; Fjerdingstad *et al.* 1998; Helmkampf *et al.* 2008) by multiplex PCR using QIAGEN multiplex PCR kit in a 10-μl reaction volume containing 5 μL Qiagen Mastermix, 1 μL primer mix containing 40 nM of each of the forward and reverse primers, 2 μL H_2O and 2 μL of template DNA. PCRs were performed in a PTC-100 thermal cycler (MJ Research) using the following program: 95 °C for 15 min, 35 cycles of 94 °C for 30 s 57 °C for 90 s and 72 °C for 60 s followed by 60 min at 60 °C. The diluted PCR products were subsequently genotyped using a MegaBACE 1000 sequencer (Amersham). We assigned eggs and workers to patrilines by manually scoring each sample with Fragment Profiler v1.2 software (Amersham).

Quantification of spermatheca contents

We first performed a pilot experiment to verify that we could accurately quantify the relative contributions of two males to a pooled sample of DNA using CM-PCR. CM-PCR involves amplifying polymorphic sites and then determining the ratio of individual-specific amplicons, which should approximate the ratio present in the template DNA. We extracted male DNA from single hind legs using the NucleoSpin Tissue extraction kit (Machery-Nagel, 740952), then measured its concentration using a Nanodrop 2000 spectrophotometer (Thermo Scientific). Samples were randomly assigned to pairs and mixed to give the following ratios of template DNA: 15:1, 7:1, 3:1, 1:1, 1:3, 1:7 and 1:15 ($n = 3$ male pairs; 198/205 mixtures successfully genotyped). Two loci (Atco12 and Atco37) were amplified separately in a PTC-100 thermal cycler as follows: Atco12: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, a 40-s annealing step of 60 °C and a 40-s extension step of 72 °C, then a final 15-min extension step at 72 °C. Atco37: 94 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, a 30-s annealing step of 55–53 °C ($\Delta t = -0.2$ °C) and a 30-s extension step, then a final

15-min extension step at 72 °C. The 10-μL reaction volumes contained 5 μL Phusion high-fidelity PCR master mix (Thermo Fisher Finnzymes), 0.5 μL each of forward and reverse primers, 3 μL H_2O and 1 μL template DNA. PCR products were genotyped using a MegaBACE 1000 sequencer, and the height and area of the peak for each allele were measured using Fragment Profiler software. The relative height and relative area of the two peaks were almost identical (Pearson's $r = 0.996$), so we opted for peak height. We used backwards stepwise removal of terms to fit the minimum adequate model of relative peak height (i.e. each datum was a pair of peaks from the same well). All interaction terms and the main effects 'locus' (i.e. Atco12 vs. Atco37) and 'relative allele size' (relative number of microsatellite repeats) did not significantly affect relative peak height (all $P > 0.25$) and were removed from the model, but the relative amount of template DNA was a strong predictor (Fig. 1; $R^2 = 0.60$, $t_{196} = 16.8$, $P < 0.0001$, $R^2 = 0.60$). The slope of the regression line was 0.96, not significantly different from 1 (95% CIs: 0.85–1.08). Some of the variation in Fig. 1 was likely caused by limitations in pipette accuracy when mixing the different ratios of template DNA, suggesting that the CM-PCR method is even more accurate than implied by these data. Based on the results of this pilot, we did not use relative allele size or locus as predictors when estimating the relative contribution of males to the stored sperm.

The spermatheca was removed from each queen by dissection. Storage in ethanol caused the sperm to form a solid mass, which could be easily separated from the queen tissue; no queen alleles were ever detected in sperm samples. The spermatheca is heart-shaped (Fig. 2), and we carefully bisected the sperm mass and extracted the contents of each lobe separately. Each DNA extract was further split in half to make a technical replicate of the CM-PCR reaction, such that there were 14 spermathecae, 28 spermathecal lobes and 56 DNA extracts. The technical replicates gave near-identical estimates of each male's contribution to the spermatheca (Pearson's r = 0.992), so we used the average of the two replicates in subsequent analyses. Sperm DNA was extracted and diluted to 20 ng/μL with ddH_2O, then amplified at four loci (Atco12, Atco13, Atco37 and Etta 5+6; $n = 224$ PCR reactions) without multiplexing (Atco13 and Etta 5+6 used the same temperature program as Atco12) and genotyped. The relative peak heights were measured as in the pilot experiment. Some peaks could not be accurately measured because one or more males possessed alleles of the same or very similar sizes. These peaks were omitted, and we estimated the relative amount of DNA from each male using the remaining loci. Of the 120 peaks that could have been measured (i.e. four loci multiplied by the number of males detected), 51 were omitted because of full or partial overlap with peaks from another male; the average number of loci used to estimate the contribution of each male was 2.3 ± 0.15. We then averaged the estimates provided by the measurable loci.

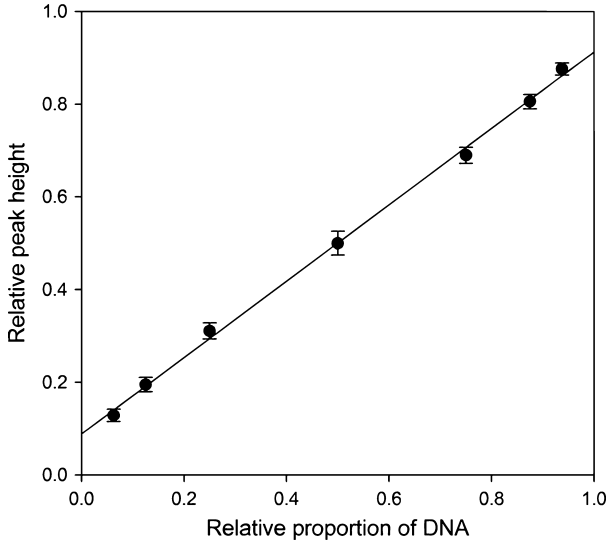

Fig. 1 Competitive PCR (CM-PCR) provided an accurate estimate of the relative abundance of two genotypes in DNA mixtures of known ratios. Points show the height of the focal individual's amplification peak as a proportion of the other male's peak, averaged across loci; data points are means ± SE.

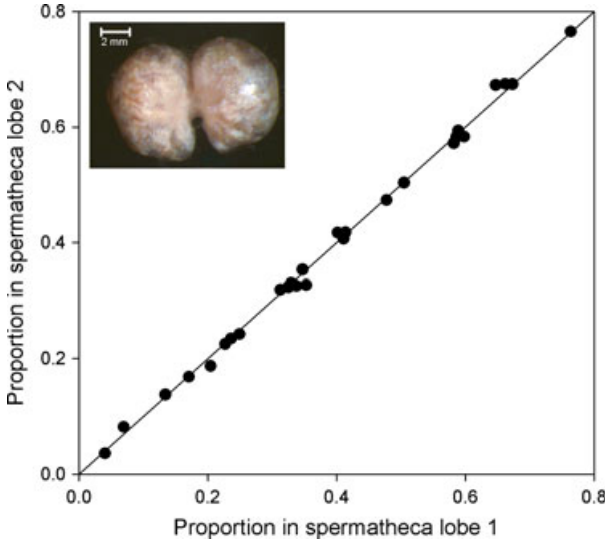

Fig. 2 Patriline distribution was near-identical between the two lobes of the spermatheca, demonstrating that sperm from different males are not spatially structured at this scale ($n = 30$ patrilines). Inset: the spermatheca of an *Atta colombica* queen containing stored sperm.

Results

The number of male genotypes detected in the sperm and offspring was the same for all colonies, i.e. we did not detect any alleles that appeared in the sperm but not the progeny and *vice versa*. The observed mating frequency was 2.29 ± 0.24 (range 1–4), and the effective mating frequency (which accounts for paternity skew; Nielsen *et al*. 2003) was 2.18 ± 0.23. The four microsatellite loci were sufficiently variable (Table S2, Supporting information) to ensure that we had a very low chance of missing patrilines because two or more males had the same multilocus genotype (nondetection error rate per colony: 0.07 ± 0.06%; Boomsma & Ratnieks 1996). Moreover, we genotyped many eggs and workers per colony (n = 85 ± 3.1), such that we had a good chance of detecting rare patrilines (1% chance of failing to detect a patriline present in 5% of the offspring; 43% chance of missing a patriline present in 1% of the offspring). Two of the 14 colonies contained only one detectable male genotype in the eggs and workers (n = 77 and 82 progeny sampled) and only one male

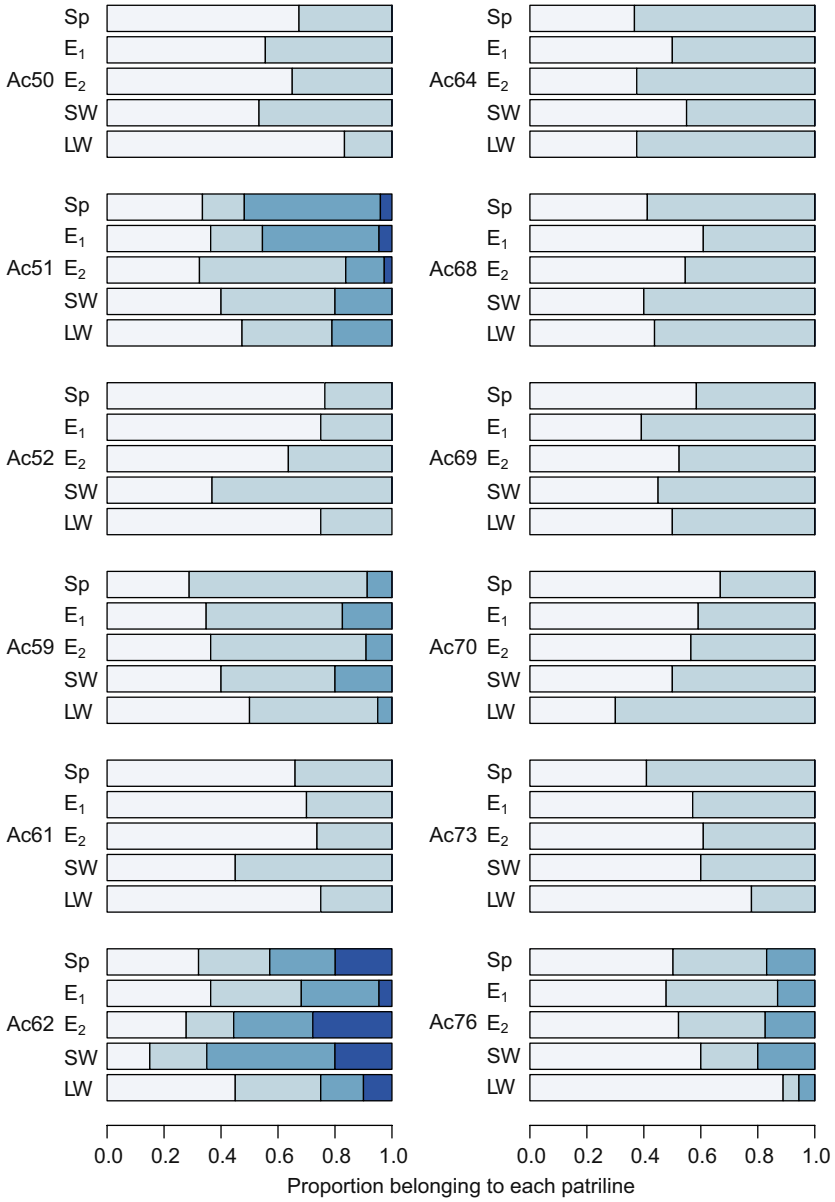

Fig. 3 Variation in patriline number and paternity skew between stored sperm, egg batches and worker castes in the 12 colonies with polyandrous queens (designated Ac50–Ac76). Each colour represents a patriline. Sp, stored sperm; E_1, first batch of eggs; E_2, last batch of eggs; SW, small workers; LW, large workers.

allele per locus in the sperm DNA, and were therefore not used in subsequent analyses. This finding confirms previous reports that some *Atta colombica* colonies are headed by a singly mated queen, at least in 1- to 2-year-old colonies (Fjerdingstad & Boomsma 1998 found that 1/22 colonies was singly mated; Helmkampf *et al.* 2008 found 2/48). Figure 3 shows the number of patrilines and their relative abundances at different life stages in the 12 colonies headed by polyandrous queens.

The estimated proportion of sperm from each male was almost identical in the two lobes of the spermatheca (Fig. 2; Pearson's $r = 0.992$), suggesting that stored sperm are thoroughly mixed and further supporting the high repeatability of the CM-PCR method. We therefore used the average estimate of the two spermathecal lobes in subsequent analyses.

The relative abundance of a male's sperm in the spermatheca was a strong predictor of the proportion of eggs he fertilized (Fig. 4; $R^2 = 0.67$, $t_{28} = 7.55$, $P < 0.0001$). To test whether the observed data fit the fair raffle model, we used simulations to generate 95% confidence limits for the relationship between estimated sperm share and estimated fertilization success under the fair raffle null model with a specified degree of measurement error. The true egg paternity was assumed to be estimated with some inaccuracy because of sampling error, such that the estimate differed from the true egg paternity by an amount ε_1, sampled from a normal distribution with $\bar{x} = 0$ and $\sigma = 0.067$ (the expected distribution of errors with $n = 43$ eggs, the mean in this study). We assumed either that the proportion of sperm was estimated without error (Fig. 4; inner CIs) or that the estimated proportion of sperm differed from the true value by an amount ε_2 (Fig. 4; outer CIs and shading). The error term ε_2 was sampled from a normal distribution with $\bar{x} = 0$ and $\sigma = 0.10$; σ was estimated from the pilot study as the mean of the within-group standard deviations and is probably an over-estimate of the true measurement error because it includes pipetting inaccuracy introduced in the pilot study. Figure 4 shows that the observed patterns of fertilization conform to the fair raffle model: there was no evidence that any patrilines had significantly more or fewer offspring than expected from their share of the sperm in storage, demonstrating that relative sperm number explains most or all of the variation in paternity.

To test whether the observed patriline distributions in large and small workers differed from those expected by chance given the observed patriline frequencies, we replicated the statistical analysis by Wiernasz & Cole (2010). Specifically, we quantified the difference in patriline distribution (Δ_{PD}) between the two worker castes

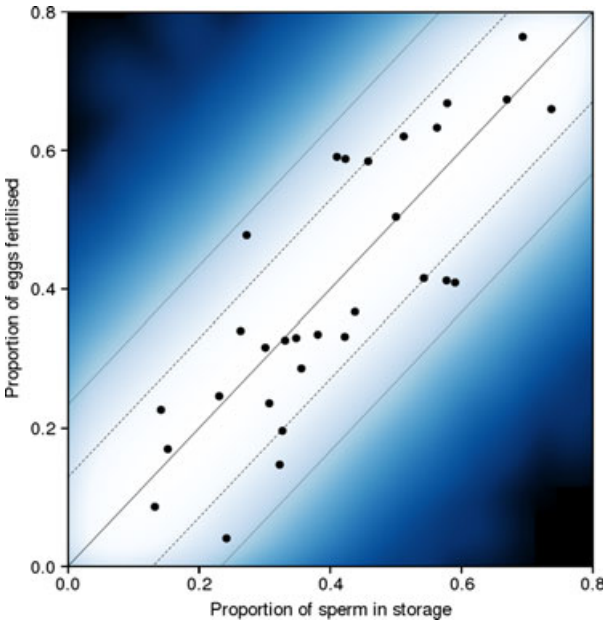

Fig. 4 The proportion of sperm in the spermatheca belonging to a given male was a strong predictor of the proportion of the eggs he fertilized. The shading shows the density of simulated data points assuming 'fair raffle' sperm competition and measurement error in both variables, with lighter areas denoting higher densities (10^6 replicates). The dotted lines show the 95% confidence intervals (CIs) around the predicted slope (solid line), assuming that the proportion of sperm was measured with error, while the dashed lines show the 95% CIs assuming measurement error in the egg data only. The observed data (black circles) suggest that fertilization success is primarily or entirely determined by the relative numbers of stored sperm.

as $\Delta_{PD} = \sqrt{a^2 + b^2 \cdots + n^2}$, where each letter represents the difference in proportion between large and small workers for each of the *a* to *n* patrilines. We then generated 10 000 dummy colonies of size *N* by randomly reassigning the labels 'large' and 'small' to the *N* workers from the *n* patrilines in the focal colony. Δ_{PD} was calculated for each dummy colony, yielding the predicted distribution of Δ_{PD} under the null hypothesis that each patriline had an equal probability of belonging to either worker type. We then calculated standardized effect size for each colony as the difference between the observed Δ_{PD} and the mean null Δ_{PD}, divided by the standard deviation of the null Δ_{PD} distribution (i.e. the difference in standard normal variate units). The mean effect size across colonies is zero if the null hypothesis is true, and positive effect sizes show that the paternity differences between worker castes are greater than expected by chance. The mean effect size was 1.40, with 95% confidence intervals of 0.28–2.54 (Fig. 5; $P < 0.01$). We therefore found strong support for the hypothesis that patrilines are nonrandomly

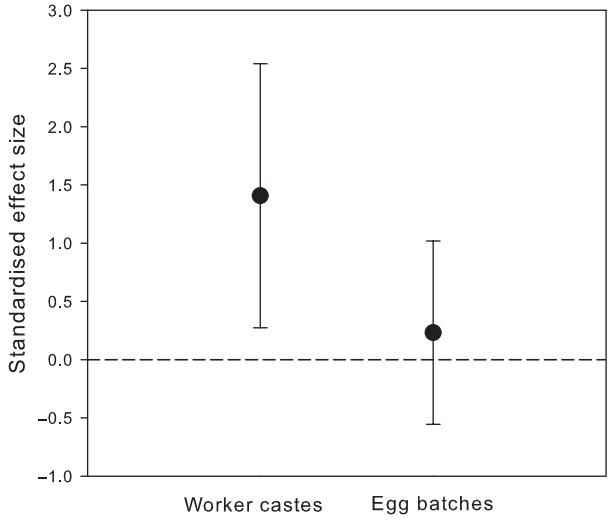

Fig. 5 The distribution of patrilines differed between worker castes more than expected by chance but did not vary between egg batches. Effect size and its 95% confidence intervals were generated by permutation (see text). These results suggest that worker caste determination has a genetic component, and sperm use does not vary significantly over time.

distributed between the two worker size classes across colonies.

We also compared the earliest batch of eggs collected with the latest (mean time between egg batches = 115 ± 6.6 days), to test whether the proportion of eggs fertilized by each male is constant over time. The statistical analysis was the same as for workers, except that we compared the paternity of the earliest and latest egg batches instead of large and small workers. The mean effect size was 0.23, with 95% confidence intervals of −0.55 to 1.02 (Fig. 5; $P > 0.4$). The data therefore do not reject the null hypothesis that patriline distribution is constant across egg batches. Together with the findings that sperm is well-mixed between the two spermathecal lobes and that fertilization success is determined by relative sperm numbers in storage, these data indicate that sperm are released from the spermatheca at random and are not physically stratified or clumped in storage.

Discussion

Our study provides a number of new insights into the mating biology of *Atta colombica*. The CM-PCR results demonstrate that sperm competition after storage is decided entirely or predominantly by variation in sperm number between competing males: there was no evidence for the involvement of other factors such as intermale variation in sperm quality (Snook 2005) or cryptic female choice (Eberhard 1996). Our results are inconsistent with the existence of genetic incompatibility between some queens and males: if certain combinations of male and female genotypes produced many inviable offspring, a deviation from the fair raffle would be expected. Evidence of cytoplasmic incompatibility, such as that introduced by *Wolbachia* in a number of nonsocial insects (Werren 1997), was similarly not detected. Stored sperm appear to be well-mixed, as the relative amount of sperm from each male did not differ between the two lobes of the spermatheca, and sperm use was not significantly different across egg batches. Sperm mixing would likely benefit queens by maximizing colony genetic diversity, but further experiments are required to determine whether sperm are mixed by active processes. The CM-PCR data also suggest that the majority of stored sperm were alive and/or that the proportion of dead sperm was comparable for all males; if some males' sperm died faster than others and remained in storage, we might have observed deviations from the fair raffle. Accordingly, a sperm viability staining study suggested that the majority (80–100%) of sperm stored by young *A. colombica* queens are alive (den Boer *et al.* 2009).

Our results shed light on the potential for sexual conflict over paternity in *A. colombica*. Shortly after mating, male seminal fluid is thought to reduce the survival of competing males' sperm, but queen spermathecal secretions apparently halt this process (den Boer *et al.* 2010). Losing sperm could be detrimental to queens, because queens' reproductive success is likely sperm-limited (den Boer *et al.* 2009) and because genetically diverse worker offspring are potentially beneficial (Hughes & Boomsma 2004; Oldroyd & Fewell 2007). However, sperm killing is predicted to select for increased sperm production by males (Greeff & Parker 2000) such that sperm limitation seems unlikely, and antagonistic interactions among ejaculates might actually increase offspring diversity. For example, first-mating males might be able to inseminate more sperm than later males but lose more sperm because they are exposed to more rival seminal fluid. Losing some sperm may also benefit queens, because sperm storage is physiologically costly and increases mortality in *A. colombica* (Baer *et al.* 2006).

Our data imply that poststorage fertilization success is determined by relative sperm numbers alone. A possible ultimate explanation for this result is the convergence of male and female reproductive interests caused by the absence of queen remating in social Hymenoptera (Boomsma *et al.* 2005). Adaptations improving success in sperm competition can only invade if the increase in paternity is not outweighed by a reduction in queen/colony fitness: for example, a 50% paternity share of 1000 offspring is better for males than 60% of

800. Male adaptations that lower their mate's survival (Morrow *et al.* 2003), cause sperm limitation (den Boer *et al.* 2009) or reduce offspring genetic diversity (Hughes & Boomsma 2004; Oldroyd & Fewell 2007; Ugelvig *et al.* 2010) may therefore be prevented from evolving. This constraint arises because any cost to offspring production is fully borne by the queen's current mate(s), rather than passed on to future mates as in species in which females mate throughout life. By contrast, male adaptations that improve fertilization success without harming colony productivity should have no such barriers to invasion. Long-lived sperm may provide one such example, because they simultaneously help male and female reproductive interests (den Boer *et al.* 2009). In short, sexual conflict over paternity is likely to be lower than in taxa with female late-life remating, which has apparently limited adaptations to sperm competition to traits that do not cause significant 'collateral damage' to females (Boomsma *et al.* 2005).

Our data provide strong evidence that worker size polymorphism has a genetic component. The distribution of patrilines differed between large and small workers more than expected by chance, suggesting that some patrilines are genetically predisposed to becoming one morph over another. Recent data on *A. colombica* showing that worker body size varies among patrilines (Evison & Hughes 2011) complement this conclusion. Patriline distribution was similar in the large and small workers in some of our colonies, but this is expected to occur even with genetic caste determination: by chance, some queens will mate with males that have a similar breeding value for worker caste. Other queens will choose males with different caste biases, leading to conspicuous paternity differences between large and small workers. Comparing the paternity of different female morphs has been used by a number of previous studies to infer genetic effects on caste fate (e.g. Hughes *et al.* 2003; Moritz *et al.* 2005; Jaffé *et al.* 2007; Hughes & Boomsma 2008), but this approach has recently been criticized. Wiernasz & Cole (2010) suggested that temporal variation in sperm use could give the illusion of genetic caste determination if castes are produced at different times of the year. Consistent with this hypothesis, the paternity of adult females varies over time in colonies of the ants *Pogonomyrmex occidentalis* (Wiernasz & Cole 2010) and *Formica truncorum* (Sundström & Boomsma 2000). However, we know of no previous studies that directly quantified temporal variation in the paternity of eggs, so it is not possible to rule out alternative hypotheses for temporal patriline variation among adults, such as genetic variation for lifespan or growth rate at different times of the year. Our study suggests that variation in egg paternity is minimal over a timescale of *c.* 5 months. This period is longer than the egg-to-adult development time (Dijkstra & Boomsma 2006), leaving genetic effects on worker development the most likely explanation for our results. Furthermore, queens are expected to be under selection to minimize paternity differences between egg batches whenever colony productivity increases with worker genetic diversity, for example by physically mixing stored sperm as implied by our data. Male traits that increase temporal variation in sperm use (e.g. sperm that clump together in storage) may also be selected against by colony-level costs, unless these traits substantially benefit males (as proposed for species with worker-controlled split sex ratios; see Sundström & Boomsma 2000).

Evidence for genetic effects on female caste fate has been found in another advanced fungus-growing ant, *Acromyrmex echinatior* (Hughes *et al.* 2003; Hughes & Boomsma 2008), in which polyandry is considerably higher (Sumner *et al.* 2004). Genetic predispositions towards particular female morphs are expected to be maintained most easily when queens are multiply mated, because the likelihood that a colony will contain only one specialization (e.g. only large worker-biased patrilines) declines exponentially with the number of queen mates, and biases towards one morph may be costly (Schwander *et al.* 2006). The discovery of substantial genetic effects on worker size therefore runs counter to this expectation, because *A. colombica* queens typically mate with a small number of males; the effect size of the caste bias in the highly polyandrous *A. echinatior* is comparable to that reported here (Wiernasz & Cole 2010). The fact that we typically detected all patrilines in both worker morphs suggests 'weak' genetic caste determination (*sensu* Anderson *et al.* 2008), in which caste fate is also strongly influenced by environmental factors. Nurse workers tending the larvae might therefore be able to control larval feeding to keep a favourable ratio of large to small workers (Linksvayer 2006; Hughes & Boomsma 2007; Schmidt *et al.* 2011), overriding innate tendencies towards particular worker morphs when necessary.

A recent study of three *A. colombica* colonies genotyped workers to estimate queen mating frequency, and obtained a higher estimate than reported here (6–7) using the same Gamboa population (Evison & Hughes 2011). Both studies estimated mating frequency from large offspring samples, suggesting the disparity arose from biological rather than methodological differences. The colonies used by Evison & Hughes (2011) were mature while ours were incipient, consistent with selective disappearance of colonies with queens that mated a small number of times. However, other factors, such as among-year variation in mating frequency caused by stochastic environmental factors (e.g. weather during the mating flight), cannot be ruled out.

The CM-PCR method employed here provided accurate measures of the ratio of sperm in a mixed sample. Counting each male's sperm with quantitative PCR would potentially be even more accurate, but this method is restrictive because each male would require at least one primer that only amplifies the DNA of that individual. Single nucleotide polymorphism (SNP) primers specific to each male are an obvious candidate, but this limits estimation of relative DNA contributions to species in which multiple SNPs have been found. By contrast, the CM-PCR method can be easily adapted to any species in which polymorphic microsatellite loci are known and could provide novel insights into previously inaccessible aspects of sperm biology. For example, many arthropods have multiple sperm storage organs, and it has been suggested that successive male ejaculates are differentially stored in each one, facilitating female control over paternity (Eberhard 1996). By analysing the contents of each storage organ, this hypothesis could be tested. Sperm have also long been suspected of physically displacing rivals from storage (Parker *et al.* 2010), but definitive demonstrations (e.g. Manier *et al.* 2010) are rare. CM-PCR provides a powerful tool for inferring which sperm belong to whom, which has previously only been possible using specialized labelling techniques (Price *et al.* 1999; Fisher & Hoekstra 2010; Manier *et al.* 2010). CM-PCR has also been used to determine relative paternity in pooled offspring DNA extracts, allowing multiple offspring to be genotyped simultaneously (Wooninck *et al.* 2000), although this application may be inaccurate if offspring size (and therefore DNA content) differs systematically between fathers.

In summary, we show that sperm use after storage is determined primarily or entirely by relative numbers, suggesting the absence of intermale differences in sperm competitive ability, cryptic female choice and genetic or cytoplasmic incompatibility. These results are consistent with predictions that sexual conflict over paternity should be constrained when females do not remate later in life. The observed random sperm use is expected to be optimal for females, suggesting either that males have no opportunity to bias or monopolize paternity after sperm has been stored and/or have no fitness interest in doing so. We also show that stored sperm are well-mixed, leading to consistent sperm use over time and provide strong evidence that worker size has a genetic component.

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References

Anderson KE, Linksvayer TA, Smith CR (2008) The causes and consequences of genetic caste determination in ants (Hymenoptera: Formicidae). *Myrmecological News*, **11**, 119–132.

Baer B (2011) The copulation biology of ants (Hymenoptera: Formicidae). *Myrmecological News*, **14**, 55–68.

Baer B, Boomsma JJ (2006) Mating biology of the leaf-cutting ants *Atta colombica* and *A. cephalotes*. *Journal of Morphology*, **267**, 1165–1171.

Baer B, Maile R, Schmid-Hempel P, Morgan ED, Jones GR (2000) Chemistry of a mating plug in bumblebees. *Journal of Chemical Ecology*, **26**, 1869–1875.

Baer B, Armitage SAO, Boomsma JJ (2006) Sperm storage induces an immunity cost in ants. *Nature*, **441**, 872–875.

Baer B, Heazlewood JL, Taylor NL, Eubel H, Millar AH (2009) The seminal fluid proteome of the honeybee *Apis mellifera*. *Proteomics*, **9**, 2085–2097.

Birkhead TR, Møller AP (1998) *Sperm Competition and Sexual Selection*. Academic Press, London.

den Boer SPA, Baer B, Dreier S, Aron S, Nash DR, Boomsma JJ (2009) Prudent sperm use by leaf-cutter ant queens. *Proceedings of the Royal Society B-Biological Sciences*, **276**, 3945–3953.

den Boer SPA, Baer B, Boomsma JJ (2010) Seminal fluid mediates ejaculate competition in social insects. *Science*, **327**, 1506–1509.

Boomsma JJ (2007) Kin selection versus sexual selection: why the ends do not meet. *Current Biology*, **17**, R673–R683.

Boomsma JJ (2009) Lifetime monogamy and the evolution of eusociality. *Philosophical Transactions of the Royal Society B-Biological Sciences*, **364**, 3191–3207.

Boomsma JJ, Ratnieks FLW (1996) Paternity in eusocial Hymenoptera. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, **351**, 947–975.

Boomsma JJ, Sundstrom L (1998) Patterns of paternity skew in *Formica* ants. *Behavioral Ecology and Sociobiology*, **42**, 85–92.

Boomsma JJ, Van der Have TM (1998) Queen mating and paternity variation in the ant *Lasius niger*. *Molecular Ecology*, **7**, 1709–1718.

Boomsma JJ, Baer B, Heinze J (2005) The evolution of male traits in social insects. *Annual Review of Entomology*, **50**, 395–420.

Bretman A, Newcombe D, Tregenza T (2009) Promiscuous females avoid inbreeding by controlling sperm storage. *Molecular Ecology*, **18**, 3340–3345.

Bussière LF, Demont M, Pemberton AJ, Hall MD, Ward PI (2010) The assessment of insemination success in yellow dung flies using competitive PCR. *Molecular Ecology Resources*, **10**, 292–303.

Dijkstra M, Boomsma J (2006) Are workers of *Atta* leafcutter ants capable of reproduction? *Insectes Sociaux*, **53**, 136–140.

Eberhard WG (1996) *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton University Press, Princeton, New Jersey.

Evison SEF, Hughes WOH (2011) Genetic caste polymorphism and the evolution of polyandry in *Atta* leaf-cutting ants. *Naturwissenschaften*, **98**, 643–649.

Fisher HS, Hoekstra HE (2010) Competition drives cooperation among closely related sperm of deer mice. *Nature*, **463**, 801–803.

Fjerdingstad EJ, Boomsma JJ (1998) Multiple mating increases the sperm stores of *Atta colombica* leafcutter ant queens. *Behavioral Ecology and Sociobiology*, **42**, 257–261.

Fjerdingstad EJ, Boomsma JJ, Thoren P (1998) Multiple paternity in the leafcutter ant *Atta colombica*—a microsatellite DNA study. *Heredity*, **80**, 118–126.

Greeff JM, Parker GA (2000) Spermicide by females: what should males do? *Proceedings of the Royal Society of London Series B-Biological Sciences*, **267**, 1759–1763.

Hall MD, Bussiere LF, Demont M, Ward PI, Brooks RC (2010) Competitive PCR reveals the complexity of postcopulatory sexual selection in *Teleogryllus commodus*. *Molecular Ecology*, **19**, 610–619.

Hamilton WD (1964) The genetical evolution of social behaviour. I and II. *Journal of Theoretical Biology*, **7**, 1–52.

Helmkampf M, Gadau J, Feldhaar H (2008) Population- and sociogenetic structure of the leaf-cutter ant *Atta colombica* (Formicidae, Myrmicinae). *Insectes Sociaux*, **55**, 434–442.

Hölldobler B, Wilson EO (1990) *The Ants*. Springer-Verlag, Berlin.

Holman L, Snook RR (2008) A sterile sperm caste protects brother fertile sperm from female-mediated death in *Drosophila pseudoobscura*. *Current Biology*, **18**, 292–296.

Hughes WOH, Boomsma JJ (2004) Genetic diversity and disease resistance in leaf-cutting ant societies. *Evolution*, **58**, 1251–1260.

Hughes WOH, Boomsma JJ (2007) Genetic polymorphism in leaf-cutting ants is phenotypically plastic. *Proceedings of the Royal Society B-Biological Sciences*, **274**, 1625–1630.

Hughes WOH, Boomsma JJ (2008) Genetic royal cheats in leaf-cutting ant societies. *Proceedings of the National Academy of Sciences of the USA*, **105**, 5150–5153.

Hughes WOH, Sumner S, Van Borm S, Boomsma JJ (2003) Worker caste polymorphism has a genetic basis in *Acromyrmex* leaf-cutting ants. *Proceedings of the National Academy of Sciences of the USA*, **100**, 9394–9397.

Jaffé R, Kronauer DJC, Bernhard Kraus F, Boomsma JJ, Moritz RFA (2007) Worker caste determination in the army ant *Eciton burchellii*. *Biology Letters*, **3**, 513–516.

King M, Eubel H, Millar AH, Baer B (2011) Proteins within the seminal fluid are crucial to keep sperm viable in the honeybee *Apis mellifera*. *Journal of Insect Physiology*, **57**, 409–414.

Linksvayer TA (2006) Direct, maternal, and sibsocial genetic effects on individual and colony traits in an ant. *Evolution*, **60**, 2552–2561.

Manier MK, Belote JM, Berben KS, Novikov D, Stuart WT, Pitnick S (2010) Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. *Science*, **328**, 354–357.

Mikheyev AS (2003) Evidence for mating plugs in the fire ant *Solenopsis invicta*. *Insectes Sociaux*, **50**, 401–402.

Miller GT, Pitnick S (2002) Sperm-female coevolution in *Drosophila*. *Science*, **298**, 1230–1233.

Moritz R, Lattorff H, Neumann P, Kraus F, Radloff S, Hepburn H (2005) Rare royal families in honeybees, *Apis mellifera*. *Naturwissenschaften*, **92**, 488–491.

Morrow EH, Arnqvist G, Pitnick S (2003) Adaptation versus pleiotropy: why do males harm their mates? *Behavioral Ecology*, **14**, 802–806.

Nielsen R, Tarpy DR, Reeve HK (2003) Estimating effective paternity number in social insects and the effective number of alleles in a population. *Molecular Ecology*, **12**, 3157–3164.

Oldroyd BP, Fewell JH (2007) Genetic diversity promotes homeostasis in insect colonies. *Trends in Ecology and Evolution*, **22**, 408–413.

Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*, **45**, 525–567.

Parker GA (1990) Sperm competition games—Raffles and roles. *Proceedings of the Royal Society of London Series B*, **242**, 120–126.

Parker GA, Immler S, Pitnick S, Birkhead TR (2010) Sperm competition games: sperm size (mass) and number under raffle and displacement, and the evolution of P-2. *Journal of Theoretical Biology*, **264**, 1003–1023.

Price CSC, Dyer KA, Coyne JA (1999) Sperm competition between *Drosophila* males involves both displacement and incapacitation. *Nature*, **400**, 449–452.

Price TAR, Hodgson DJ, Lewis Z, Hurst GDD, Wedell N (2008) Selfish genetic elements promote polyandry in a fly. *Science*, **322**, 1241–1243.

Schmidt AM, Linksvayer TA, Boomsma JJ, Pedersen JS (2011) Queen-worker caste ratio depends on colony size in the pharaoh ant (*Monomorium pharaonis*). *Insectes Sociaux*, **58**, 139–144.

Schwander T, Rosset H, Chapuisat M (2005) Division of labour and worker size polymorphism in ant colonies: the impact of social and genetic factors. *Behavioral Ecology and Sociobiology*, **59**, 215–221.

Schwander T, Cahan SH, Keller L (2006) Genetic caste determination in *Pogonomyrmex* harvester ants imposes costs during colony founding. *Journal of Evolutionary Biology*, **19**, 402–409.

Schwander T, Lo N, Beekman M, Oldroyd BP, Keller L (2010) Nature versus nurture in social insect caste differentiation. *Trends in Ecology and Evolution*, **25**, 275–282.

Simmons LW (2001) *Sperm Competition and Its Evolutionary Consequences in the Insects*. Princeton University Press, Princeton, New Jersey.

Snook RR (2005) Sperm in competition: not playing by the numbers. *Trends in Ecology and Evolution*, **20**, 46–53.

Sumner S, Hughes WOH, Pedersen JS, Boomsma JJ (2004) Ant parasite queens revert to mating singly. *Nature*, **428**, 35–36.

Sundström L, Boomsma JJ (2000) Reproductive alliances and posthumous fitness enhancement in male ants. *Proceedings of the Royal Society B-Biological Sciences*, **267**, 1439–1444.

Ugelvig LV, Kronauer DJC, Schrempf A, Heinze J, Cremer S (2010) Rapid anti-pathogen response in ant societies relies on high genetic diversity. *Proceedings of the Royal Society B-Biological Sciences*, **277**, 2821–2828.

Van Borm S, Wenseleers T, Billen J, Boomsma JJ (2001) *Wolbachia* in leafcutter ants: a widespread symbiont that may

induce male killing or incompatible matings. *Journal of Evolutionary Biology*, **14**, 805–814.
Wenseleers T (2007) Nepotism absent in insect societies—or is it? *Molecular Ecology*, **16**, 3063–3065.
Werren JH (1997) Biology of *Wolbachia*. *Annual Review of Entomology*, **42**, 587–609.
Wiernasz DC, Cole BJ (2010) Patriline shifting leads to apparent genetic caste determination in harvester ants. *Proceedings of the National Academy of Sciences of the USA*, **107**, 12958–12962.
Wilhelm M, Chhetri M, Rychtar J, Rueppell O (2011) A game theoretical analysis of the mating sign behavior in the honey bee. *Bulletin of Mathematical Biology*, **73**, 626–638.
Wilson EO (1980) Caste and division of labour in leaf-cutter ants (Hymenoptera, Formicidae, Atta).1. The overall pattern in Atta sexdens. *Behavioral Ecology and Sociobiology*, **7**, 143–156.
Wooninck LM, Warner RR, Fleischer RC (2000) Relative fitness components measured with competitive PCR. *Molecular Ecology*, **9**, 1409–1414.

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Data accessibility

Multilocus microsatellite genotypes of all adults and eggs are archived at Dryad, doi: 10.5061/dryad.jf803j0r.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Sample sizes and collection dates of different offspring types successfully genotyped for paternity analysis.

Table S2 Description of microsatellite loci used in the present study (Fjerdingstad *et al*. 1998; Helmkampf *et al*. 2008).

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