Behavioral Ecology
doi:10.1093/beheco/arn112
Advance Access publication 11 September 2008

Sperm length evolution in the fungus-growing ants

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Eusocial insects offer special opportunities for the comparative study of sperm traits because sperm competition is absent (in species with obligatory monandry) or constrained (in lineages where queens mate multiply but never remate later in life). We measured sperm length in 19 species of fungus-growing ants, representing 9 of the 12 recognized genera, and mapped these onto the ant phylogeny. We show that average sperm length across species is highly variable and decreases with mature colony size in basal genera with singly mated queens, suggesting that sperm production or storage constraints affect the evolution of sperm length. Sperm length does not decrease further in multiply mating leaf-cutting ants, despite substantial further increases in colony size. In a combined analysis, sexual dimorphism explained 63.1% of the variance in sperm length between species. As colony size was not a significant predictor in this analysis, we conclude that sperm production trade-offs in males have been the major selective force affecting sperm length across the fungus-growing ants, rather than storage constraints in females. The relationship between sperm length and sexual dimorphism remained robust in phylogenetically independent contrasts. Some of the remaining variation was explained by the relative size of the sperm-storage organ, but only in the multiply mating leaf-cutting ants, suggesting that sperm-storage constraints become important for the evolution of sperm length in this derived group. Mate number affected sperm length to a minor extent, and only in interaction with other predictor variables, suggesting that sperm competition has not been a major selective force for sperm length evolution in these ants. *Key words:* sexual selection, social insects, sperm limitation, sperm-storage constraints. *[Behav Ecol 20:38–45 (2009)]*

INTRODUCTION

Postcopulatory sexual selection has repeatedly been hypothesized to drive evolutionary change in sperm length (Gage 1998; Snook 2005; Beese et al. 2006; Garcia-Gonzalez and Simmons 2007). Early theoretical studies predicted that selection should favor the production of numerous, small sperm (Parker 1982; Parker and Begon 1993) if sperm number determines the outcome of sperm competition (for some empirical evidence, see Martin et al. [1974] and Gage and Morrow [2003]). However, the evolution of long sperm has also been predicted to be advantageous, if longer sperm moves faster, lives longer, or is used by the female as an indicator of male quality (see review by Snook [2005]). Empirical studies that tested the effects of sperm length on the outcome of sperm competition for paternity success have reported mixed results. In phylogenetic comparisons, longer sperm was associated with increased levels of sperm competition in cichlid fish (Balshine et al. 2001), frogs (Byrne et al. 2003), and moths (Morrow and Gage 2000). However, no correlation (Briskie and Montgomerie 1992; Hosken 1997; Gage et al. 2004) or negative correlations (see e.g., Gage and Freckleton 2003; Garcia-Gonzalez and Simmons 2007) between sperm competition and sperm length have been found in a number of other comparative and experimental studies on sperm length. These contrasting patterns suggest that sperm length may be affected by different sets of variables across lineages (for an example of female influence on sperm length evolution, see Miller and Pitnick 2002) so that the overall functional significance of sperm length variation has remained a conundrum to evolutionary biologists (Snook 2005). This may be because the forces of sexual selection that affect male and female fitness as a function of sperm length are only partially understood or because the evolution of sperm traits is affected by production and storage constraints, an issue that has rarely been addressed (e.g., Briskie and Montgomerie 1992).

Ants are a highly suitable group for studying constraints on sperm length evolution. Female ants (queens) never remate after a single brief episode of nuptial flights early in adult life so that reproductive success in species with long-lived queens and large mature colonies is ultimately limited by the amount of sperm initially stored (Weber 1972; Pamilo 1991). Across species or genera, we thus expect sperm length to decrease with increasing colony size to permit packing of a greater number of sperm into the females' sperm-storage organ (Boomsma et al. 2005), but so far no comparative studies have tested this hypothesis. Another key trait of almost all ant males is that spermatogenesis ends shortly after eclosion (Heinze and Hölldobler 1993) so that male lifetime production of sperm is fixed and dependent on larval nutrition only, leading to a direct trade-off between sperm length and sperm number.

Obligate polyandry has evolved as a derived trait in some lineages of ants, bees, and wasps (Boomsma and Ratnieks 1996; Strassmann 2001; Baer 2005; Kronauer et al. 2007) so that sperm competition for storage or egg fertilization may have shaped sperm length in these lineages. The few studies available indicate that males of bees and ants have longer sperm in

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Received 10 March 2008; revised 11 June 2008; accepted 5 August 2008.

species with multiply mated queens compared with their singly mated sister taxa (Lino Neto and Dolder 2002; Baer et al. 2003; Baer 2005; Baer and Boomsma 2006). However, these comparisons were based on very few species, were not controlled for phylogenetic correlations, and were not related to a larger set of predictor variables.

The present study investigates sperm length variation across 19 species (representing 9 of the 12 recognized genera) of attine fungus-growing ants and maps the average sperm lengths onto the attine phylogeny (Villesen et al. 2002; Sumner, Aanen, et al. 2004). The attine ants are one of the more interesting tribes of eusocial insects for such a comparative analysis because there is large variation across genera in size and longevity of mature colonies and in size, fecundity, and mating frequency of queens (Table 1). This implies that sperm-storage constraints may have become more acute during the evolutionary transition from the basal genera with small, short-lived colonies of less than 100 workers to the highly advanced leaf-cutting ants with millions of workers per colony and queens that may live for decades (Weber 1972). We therefore test the hypothesis that shorter sperm evolved with increasing colony size and whether mate number affects this relationship.

Sperm production and storage constraints will depend on several factors. Male size is expected to be important as it will ultimately determine the amount of sperm that can be produced and contained while still allowing males to embark on mating flights (Fjerdingstad and Boomsma 1997). Queen size is also expected to be important as it will constrain the amount of sperm that can be stored, although the upper limit to sperm storage may be better described by the size of a queen's sperm-storage organ (spermatheca) relative to her body size. Sexual dimorphism, however it has evolved, is also expected to be important in the evolution of sperm length and number because this is expected to reflect the relative resources available to males and females for sperm production and maintenance and the relative size constraints on sperm storage. Hence, we also analyze whether the absolute body size of queens and males, sexual dimorphism, and the relative size of the spermatheca can explain variation in sperm length.

MATERIALS AND METHODS

Ant sampling

Males of 16 species of fungus-growing ants were collected by excavating nests in Gamboa, Republic of Panama in May and June 2001–2004. Males were either sampled during excavation or after colonies without males were kept in the laboratory in Copenhagen at 25 °C and approximately 70% humidity until they produced males. Colonies of 3 additional species were collected in northern Argentina (*Mycetophylax emeryi* and *Mycetarotes parallelus*) and the United States (*Mycetosoritis hartmanni*) and were kept at the University of Texas at Austin until they produced males. Sperm was collected using an experimental protocol described in detail elsewhere (Baer and Schmid Hempel 2000; Baer and Boomsma 2004, 2006). In short, males were killed and then dissected with watchmaker forceps under a stereomicroscope at ×5–62.5 magnification. Both accessory testes were transferred to a microscope slide and punctured, after which the outflowing sperm was smeared over the slide and air dried. For the larger *Acromyrmex* and *Atta* males, we dissected only the right accessory testis and punctured its distal end. We then collected a subsample of the sperm with a glass capillary, which we normally use to artificially inseminate bumblebees (Baer and Schmid Hempel 2000). Earlier work in attine ants (Baer and Boomsma 2006) and bumblebees (Baer et al. 2003) indicated that sperm length does not differ between the left and the right accessory testis or among different parts of a single accessory testis.

To measure sperm length, we used a phase-contrast microscope connected to a digital camera. Slides were inspected at ×400 magnification and digital phase-contrast microscope images of single, noncoiled, intact sperm were collected. Sperm was measured by analyzing the digital images using the program National Institute of Health (NIH) Image for Macintosh OS 9.1 (freely available at http://rsb.info.nih.gov/

Table 1
The data used for the analyses

Species	No. of colonies	No. of males	Sperm length ± SD in μm	Male size ± SD in mm	Queen size ± SD in mm	Spermatheca radius ± SD in mm	Colony size	Queen mating frequency
Apterostigma sp. 2	1	5	230.49	0.96	1.09	0.082 ± 0.004	25	1
Apterostigma collare	2	37	226.01 ± 4.48	0.97 ± 0.03	0.91 ± 0.15	0.083 ± 0.007	50	1
Apterostigma dentigerum	4	37	138.06 ± 7.23	0.93 ± 0.04	1.14 ± 0.06	0.096 ± 0.016	25	1
Mycetarotes parallelus	3	28	163.58 ± 2.43	0.86 ± 0.03	0.95	0.146 ± 0.007	110	1
Mycetosoritis hartmanni	3	27	139.76 ± 2.63	0.74 ± 0.01	0.77	0.087	50	1
Cyphomyrmex costatus	1	14	106.68	0.50	0.58 ± 0.04	0.055 ± 0.004	200	1
Cyphomyrmex rimosus	3	28	104.50 ± 2.43	0.63 ± 0.02	0.65 ± 0.05	0.118 ± 0.014	150	1
Mycetophylax emeryi	1	11	80.17	0.64	—	—	100	1
Trachymyrmex cf. *zeteki*	5	102	100.17 ± 1.68	0.86 ± 0.02	1.29 ± 0.04	0.089 ± 0.004	300	1
Trachymyrmex cornetzi sp. 1	2	40	98.33 ± 1.17	0.78 ± 0.06	1.06 ± 0.01	0.079 ± 0.009	300	1
Trachymyrmex sp. 3	5	102	87.65 ± 4.90	0.70 ± 0.01	1.22 ± 0.02	0.090 ± 0.004	1000	1
Sericomyrmex cf. *amabilis*	4	82	76.34 ± 3.31	0.73 ± 0.02	1.22 ± 0.00	0.130 ± 0.010	2000	1
Sericomyrmex amabilis	2	40	74.40 ± 2.47	0.80 ± 0.01	1.29 ± 0.02	0.138 ± 0.007	2000	1
Acromyrmex octospinosus	5	101	84.32 ± 1.70	1.82 ± 0.03	2.94 ± 0.03	0.378 ± 0.008	40000	6.1
Acromyrmex echinatior	5	102	83.87 ± 0.86	1.73 ± 0.05	2.67 ± 0.04	0.364 ± 0.008	40000	9.3
Acromyrmex insinuator	2	41	82.91 ± 0.54	1.56 ± 0.03	2.38 ± 0.03	0.304 ± 0.007	100	1.15
Atta sexdens	3	56	67.06 ± 1.07	2.46 ± 0.07	4.47 ± 0.08	1.749 ± 0.245	5000000	2.7
Atta colombica	5	98	161.16 ± 2.69	2.73 ± 0.08	4.31 ± 0.08	2.206 ± 0.108	2500000	3
Atta cephalotes	5	100	150.49 ± 2.50	3.17 ± 0.06	4.88 ± 0.04	2.727 ± 0.040	3000000	≥2

Sperm length is based on mean values per colony. Male and queen size was measured as the maximum distance between and including the compound eyes. The estimates of colony size (average number of workers in mature colonies) have been collected from the literature (e.g., Weber 1972; Solomon et al. 2004) and were supplemented by our own field data. Effective queen mating frequencies were taken or inferred from the literature (Villesen et al. 2002; Sumner, Hughes, et al. 2004; see text for details). SD, standard deviation.

nih-image). We measured total sperm length for 10 sperm per male for up to 20 males per colony and for a maximum of 5 colonies per species (Table 1). Male body size was estimated by measuring head width as the maximal distance between and including the compound eyes. This measurement was used in earlier work and is a reliable indicator for body size in leaf-cutting ants (Fjerdingstad and Boomsma 1997; Baer and Boomsma 2004). A dissecting microscope with an ocular grid was used for these measurements at ×7.8–62.5 magnification.

Estimates for mature colony size were obtained from the literature, supplemented by data on colony sizes from our field notes. Colony size estimates were log transformed prior to statistical analyses to minimize sampling errors so that systematic underestimations, for example, because of escaped workers become evenly distributed over the data set. Effective queen mating frequencies for the species used were available from the literature (Villesen et al. 2002, Sumner, Hughes, et al. 2004). There are 3 broad categories of mating system in the eusocial Hymenoptera: obligatory single mating, facultative multiple mating, and obligatory multiple mating (Boomsma and Ratnieks 1996). Transitions between these categories are uncommon so that mating systems tend to be conserved within genera or larger monophyletic groups (Hughes et al. 2008), except for very rare reversals from obligatory multiple mating to facultative multiple mating associated with major shifts in life history (Sumner, Hughes, et al. 2004; Kronauer and Boomsma 2007). We therefore assumed that queen mating frequency was one in *M. emeryi*, *M. parallelus*, and *M. hartmanni*, similar to all other lower attine ants investigated so far (Villesen et al. 2002). Because of the close similarities of the sexual organs of *Atta cephalotes* and *Atta colombica* (Baer and Boomsma 2006), we also assumed that the mating frequency was similar between these 2 species, but we kept the estimate conservatively low (≥2). To estimate the volume of queen spermathecae up to 5 queens per species were collected by excavating entire colonies in the field. The queens were killed and their spermathecae dissected under a stereomicroscope at magnifications between ×12.5 and ×64. Pictures of single, undamaged spermathecae were taken with a digital camera, and the maximum circular cross section area was measured using the NIH image program. Given that spermathecae of attine ants are approximately spherical (see Figure 1), we then calculated the radius for each spermatheca based on this area.

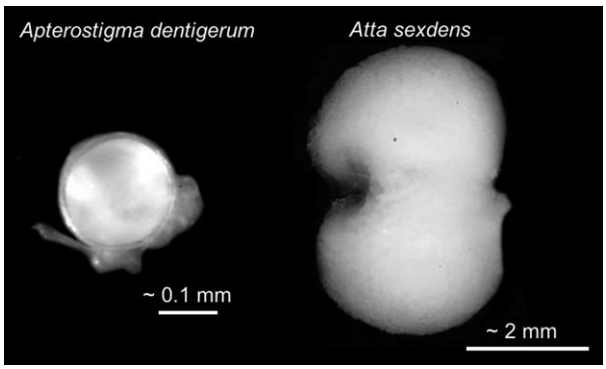

Figure 1
Two examples of spermathecae from a lower and a higher fungus-growing ant that were used to measure spermatheca size. Digital pictures were taken of dissected spermathecae. We then used the NIH program to measure the total area of the spermatheca and approximated the radius as $r = \sqrt{\text{area}/\pi}$.

Statistical analysis

Our final data set had sperm length measurements of 1051 males, originating from 61 colonies and 19 species (Table 1). The general sperm morphology was very similar across species and comparable to sperm in other eusocial Hymenoptera (Jamieson et al. 1999; Baer et al. 2003; Baer 2005). Data were analyzed with SPSS 10 and JMP 7.0.1 for Macintosh, using nested analysis of variance (ANOVA) and multiple regression models. For cross-species comparisons, sperm length, colony size, and body size were $\log_{10}$ transformed to improve the normality of the distribution of these variables and because we expected allometric relationships between them.

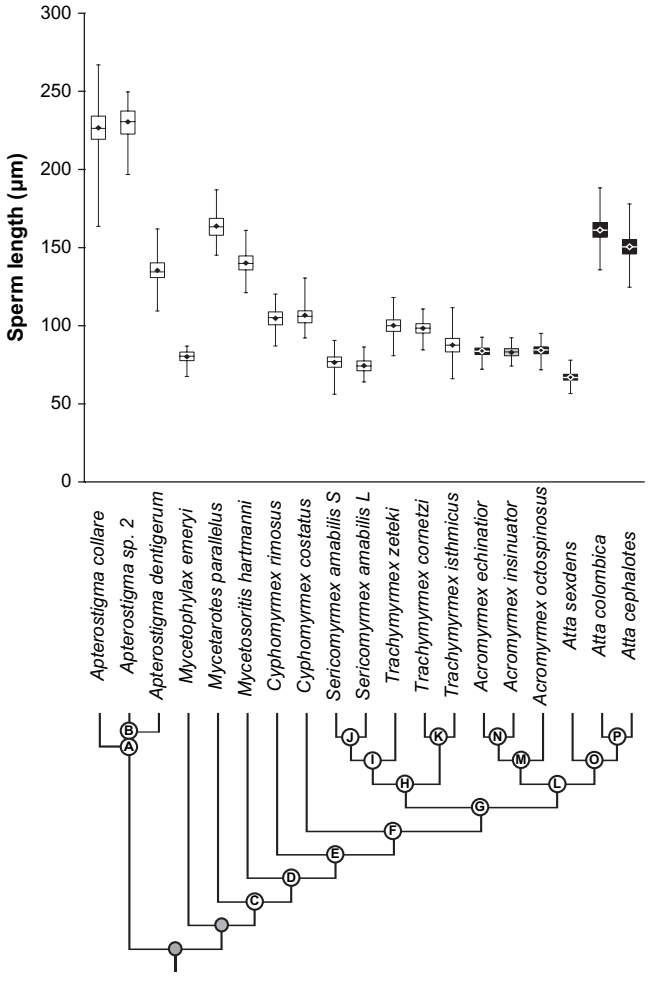

Figure 2
Sperm length of the different species of fungus-growing ants shown as box-and-whiskers plots. For each species, the overall mean sperm length is marked as a diamond and the median as a horizontal line. The lower and upper ends of each box represent the 25% and 75% quartiles of the data, respectively, and the whiskers show the entire range of the data. Unfilled boxes represent the singly mating species, whereas the multiply mating leaf-cutting ants are shown with solid boxes. The inquiline social parasite *Acromyrmex insinuator* that has secondarily reverted to single mating is shown with a gray box. Sample sizes were 5–22 males per colony and up to 5 colonies per species (for further details, see Table 1). The phylogenetic tree under the graph is based on Villesen et al. (2002), Sumner, Aanen, et al. (2004) and Schultz and Brady (2008). Lettered nodes represent those used to determine phylogenetically independent contrasts in sperm length and sexual dimorphism (contrasts could not be computed for the gray nodes—see Table 1).

All test results are given with 2-tailed probabilities. Because comparative data can be confounded by phylogenetic correlations, we also repeated our analysis using phylogenetically independent contrasts calculated in the Phenotypic Diversity Analysis Programs (PDAP) module (Midford et al. 2007) of the program Mesquite (Maddison WP and Maddison DR 2007) and a phylogeny based on published trees of Villesen et al. (2002), Sumner, Aanen, et al. (2004), and Schultz and Brady (2008). The relationships of species not specifically included in these published trees were inferred from their taxonomic similarity to included species. We had no queen size or spermatheca size data for *M. emeryi*, so this species was excluded from the analysis based on independent contrasts (Midford et al. 2007) and from those multiple regression models involving queen size or spermatheca size.

RESULTS

The mean length of sperm varied more than 3-fold across species, ranging from 67.5 to 230.5 μm (Figure 2; Table 1). Nested ANOVA showed that sperm length differed between males from the same colony ($F_{989,9459} = 6.39$, $P < 0.0001$; explaining 0.63% of the variance), between colonies of the same species ($F_{43,989} = 9.59$, $P < 0.0001$; explaining 0.38% of the variance), and between species ($F_{18,43} = 800.26$, $P < 0.0001$; explaining 97.8% of the variance). Repeating the analysis for each species separately, to examine consistency of the variation between males and colonies, showed that between-male variation in sperm length was highly significant in every species (all $P < 0.008$) and that between-colony variation was significant in 13 of the 15 species for which samples were available from more than one colony. The remaining 2 species, which had only 2 or 3 analyzed colonies, showed levels of between-colony variation in sperm length suggesting a trend in this direction (*M. parallelus*: $F_{2,25} = 1.97$, $P = 0.16$; *Acromyrmex insinuator*: $F_{1,39} = 2.31$, $P = 0.14$). For the 2 species, where males from field and laboratory-reared colonies could be compared, rearing conditions did not influence sperm length (*Apterostigma dentigerum*: $F_{1,2} = 1.55$, $P = 0.336$; *Acromyrmex echinatior*: $F_{1,3} = 0.349$, $P = 0.596$), and males were not significantly different in size in *A. dentigerum* ($F_{1,2} = 1.42$, $P = 0.355$), but on average, 5.5% smaller in laboratory-reared colonies of *A. echinatior* ($F_{1,3} = 10.46$, $P = 0.048$.). For those species in where males were collected in either the field or the laboratory, but not both, there was no relationship between the origin of the males and their size relative to queens of the same species ($F_{1,12} = 0.060$, $P = 0.811$), suggesting that smaller size among laboratory-reared males is not a consistent effect. Addition of origin of males as a variable to subsequent analysis showed no evidence of significant explanatory power, so we are confident that the different rearing conditions of different species have not affected our conclusions.

Measurements of colony size, male size, queen size, spermatheca size, and mating frequency were highly positively correlated across species (Table 2), showing that as average colony sizes increase, there are corresponding increases in queen size, male size, spermatheca size, and mating frequency. As predicted, there was a significant decrease in sperm length with increasing colony size for the singly mated species ($r = -0.80$, $F_{1,11} = 18.97$, $P = 0.001$) but no such relationship for the multiply mated species ($r = +0.40$, $F_{1,4} = 0.76$, $P = 0.432$). However, the combination of colony size, mating frequency, and their interaction only explained 43.1% of the variability in sperm length across all species, whereas other combinations of variables explained a much higher proportion of variance, for example, the simpler model combining male size and queen size explained 64.9% of the variability in sperm length.

Because queen size, male size, and spermatheca size are highly correlated (Table 2), there is a problem of multicollinearity in any multiple regression model where these variables are compared. To overcome this, we used principal component analysis to transform the $\log_{10}$ male and queen body size data into 2 uncorrelated components. The first principal component (PC1) gives the overall body size of reproductives, explained 98.2% of the combined variation in body size of males and queens, and was perfectly correlated with the mean of the body sizes of males and queens for each species. The second principal component (PC2), explaining the remaining 1.8% of the variance, provides a measure of sexual dimorphism, with positive values representing cases in which males were larger than expected relative to queens and negative values cases where males were smaller than expected (this is equivalent to using orthogonal residuals from a reduced major axis regression of male size on queen size). The correlation between spermatheca size and body size was then removed by using the residuals from a linear regression of $\log_{10}$ spermatheca size on $\log_{10}$ queen size to give a relative spermatheca size (positive values representing spermathecae that were larger than average for a given queen size and negative values those smaller than average). Body size (PC1) was highly correlated with both colony size and effective queen

Table 2

Pairwise correlation matrix for the estimates in Table 1, plus the data processed to remove correlations between size variables (see text for details)

	Original data				Processed data		
	$\log_{10}$ male size (a)	$\log_{10}$ queen size (b)	$\log_{10}$ spermatheca radius	$\log_{10}$ colony size	Body size (PC1)	Sexual dimorphism (PC2)	Relative spermatheca size
$\log_{10}$ queen mating frequency	0.782	0.783	0.734	0.782	0.788	0.005	0.014
$\log_{10}$ male size (a)		0.964	0.941	0.821	0.987	0.163	0.121
$\log_{10}$ queen size (b)			0.931	0.872	0.994	−0.107	0.000
$\log_{10}$ spermatheca radius				0.914	0.943	0.066	0.366
$\log_{10}$ colony size					0.858	−0.182	0.281
Body size (PC1)						0.000	0.049
Sexual dimorphism (PC2)							0.452

Correlation coefficients marked in bold are significantly different from zero (all $P < 0.005$). Because we have no queen and spermatheca size data for *Mycetophylax emeryi*, this species only occurs in correlations between mating frequency, male size, and colony size ($n = 19$), for all other combinations, $n = 18$.

mating frequency, but sexual dimorphism (PC2) and relative spermatheca size were uncorrelated with either (Table 2). Relative spermatheca size was also uncorrelated with sexual dimorphism (Table 2).

These transformed variables were used to replace male and queen body size and spermatheca size in multiple linear regression models, examining the effect of these 3 variables (PC1, PC2, and relative spermatheca size), colony size, and mating frequency (coded either as number of estimated fathers or as monandrous/polyandrous). Because we had a priori reason to expect that the selective pressures on monandrous and polyandrous species may be different, we also included interaction terms between mating frequency and the other variables. The interpretation of multiple regression models can be difficult (Johnson and Omland 2004; Whittingham et al. 2006) because different combinations of variables can explain similar amounts of variance, but it was clear that the single variable that explained most variance in sperm length was sexual dimorphism, which on its own explained 63.1% of the variability in sperm length between species (Figure 3; $r = +0.79$, $F_{1,16} = 27.4$, $P < 0.001$). This was also the only term that was significant when the full model was fitted to the data (cf. Whittingham et al. [2006]; $F_{1,8} = 11.62$, $P = 0.009$).

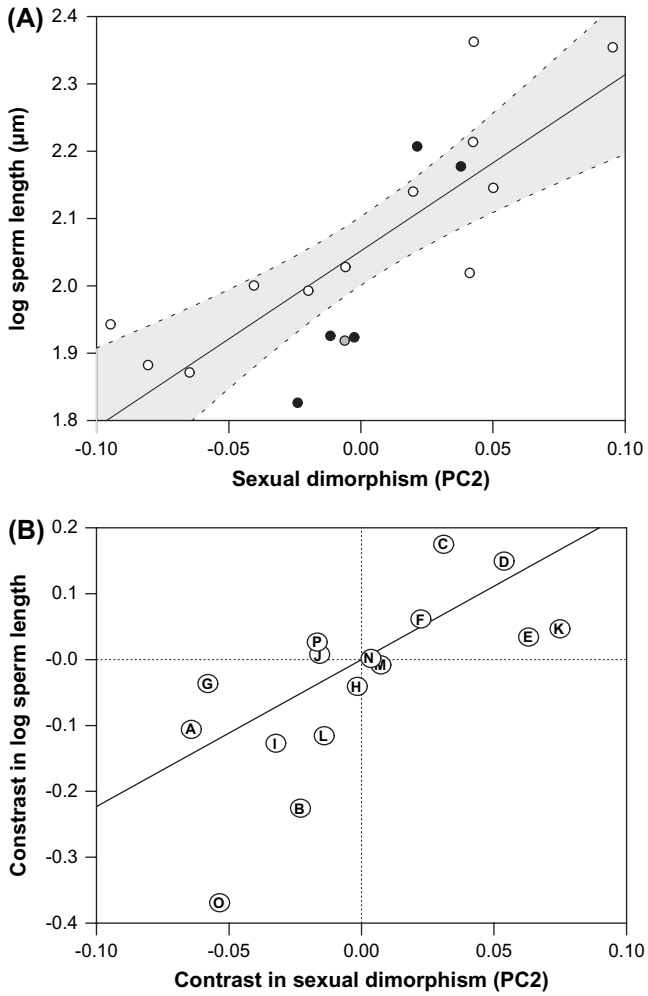

Figure 3
(A) The relationship between log sperm length and sexual dimorphism (measured as the second principal component in a PC analysis of the log of male and queen size; see text for details) for each of the species included in the analysis. The line represents a linear regression, surrounded by its 95% confidence limits. The multiply mating leaf-cutting ants are plotted as solid dots and the singly mating species as open dots. The inquiline social parasite *Acromyrmex insinuator* that has secondarily reverted to single mating has been given a gray dot. (B) The phylogenetically corrected relationship between sperm length and sexual dimorphism. Each point represents a node in the phylogenetic tree (Figure 2) for which independent contrasts in both sperm length and sexual dimorphism (Table 1) could be calculated (marked with a capital letter as shown in Figure 2). The line represents a linear regression through the origin.

All possible models combining the variables were also fitted (511 models in total), and the adequacy of their explanatory power examined by using the small sample unbiased Akaike's information criterion (Johnson and Omland 2004). A 95% confidence set of models was then calculated based on Akaike weights (Johnson and Omland 2004, Whittingham et al. 2005), giving a total of 95 models explaining between 78% and 90% of the variance in sperm length when mating frequency was treated as a binary variable and 128 models explaining between 63% and 90% of the variation in sperm length when it was treated as a continuous variable. All models in the 95% confidence set contained sexual dimorphism as a main factor (Table 3), and, as expected, the sum of Akaike weights for this variable was 0.95 (the sum of Akaike weights can be interpreted as the probability that variable is a component of the best-fitting model based on the variables used, with maximum value of 0.95 in the 95% confidence set).

Of the remaining variables, relative spermatheca size appeared more frequently than expected and significantly so in its interaction with mating frequency (Table 3). When this relationship is examined on its own, there is no correlation between relative spermatheca size and sperm length in monandrous species ($r = +0.028$, $F_{1,10} = 0.065$, $P = 0.804$), but a positive correlation in polyandrous species, albeit not reaching significance ($r = +0.679$, $F_{1,4} = 3.42$, $P = 0.138$). In this latter analysis, *Atta sexdens* is a clear outlier, having much shorter sperm than expected based on the relative size of the queen spermatheca (without *A. sexdens*, $r = +0.991$, $F_{1,3} = 199.61$, $P = 0.001$).

Mean body size (PC1) and colony size seem to have very little direct influence on sperm length, appearing in few models as either main effects or interactions. Effective queen mating frequency as a main effect also had little association with sperm length, but it did appear in a large number of the models in the interaction terms (Table 3). When effective queen mating frequency was treated as a binary variable, the number of terms in the 95% confidence set of models ranged from 2 to 6, with the simplest model consisting of sexual dimorphism and the interaction between effective queen mating frequency and relative spermatheca size. When effective queen mating frequency was treated as a continuous variable, the number of terms in the models in the 95% confidence set ranged from 1 to 7, the simplest model consisting of sexual dimorphism on its own, followed by the 2-term models incorporating sexual dimorphism in combination with effective queen mating frequency, relative spermatheca size, and their interaction.

Phylogenetically independent contrasts and multiple regression through the origin (Garland et al. 1993; see also Materials and methods) showed that the only variable that was significantly correlated with sperm length after phylogenetic patterns were removed was sexual dimorphism (PC2) (Figure 3b; $r = 0.647$, $P = 0.004$). This result was independent of the specific branch lengths used in the phylogenetic tree, and because some lengths were estimated from taxonomic similarity rather than measured, this analysis was based on an ultrametricized tree that reflected only phylogenetic topology (Figure 2). The difference between this analysis and the previous one (which did not correct for phylogenetic correlations; Figure 3a) arises because there is a general increase in the correlated variables body size, colony size, spermatheca size, and mating

Table 3

Summary of the 95% confidence sets of models based on multiple linear regression models, treating mating frequency either as a binary variable (monandrous/polyandrous) or as a continuous variable (effective queen mating frequency)

	Mating frequency binary (95 models)		Mating frequency continuous (128 models)	
Model component	No. of models	Sum of Akaike weights	No. of models	Sum of Akaike weights
Mating frequency	43	0.389	53	0.524
$\log_{10}$ colony size	37	0.313	47	0.227
Body size (PC1)	39	0.330	47	0.236
Sexual dimorphism (PC2)	95***	0.950	128***	0.949
Relative spermatheca size	31	0.172	66	0.663
Mating frequency × $\log_{10}$ colony size	35	0.184	47	0.254
Mating frequency × body size (PC1)	36	0.255	48	0.491
Mating frequency × sexual dimorphism (PC2)	54	0.468	78*	0.782
Mating frequency × relative spermatheca size	76***	0.870	68	0.311

The number of models within the 95% confidence set that contain each component is given, plus the sum of the Akaike weights for those models. Terms that appear more frequently than expected by chance, based on 1-tailed Fisher exact tests, are marked *($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$)

frequency from the lower attines to the leaf-cutting ants so that much of the effect of these on sperm length appears as a phylogenetic effect. Sexual dimorphism, on the other hand, shows no such phylogenetic pattern and turns out to be the overall key predictor variable.

DISCUSSION

Sperm length in fungus-growing ants is a highly variable trait (Figure 2), as has been found in other insects (Ward 1998; Morrow and Gage 2001; Baer et al. 2003; Gage and Freckleton 2003; Baer 2005). Our statistical analysis was able to explain a large portion (63.1%) of the variance in sperm length between species with a single variable, sexual dimorphism, that is, sperm is shorter in species with males that are small relative to queens (Figure 3a), suggesting that sperm production constraints in males may be of primary importance in determining sperm length, rather than sperm-storage constraints in females. That is, for a given queen size, smaller bodied males can produce a smaller total mass of sperm than large bodied males so that to produce the same number of sperm, smaller (shorter) sperm must be produced. These production constraints may arise for several reasons; there may be constraints on the resources that smaller males can accrue during development, smaller males will also have relatively smaller accessory testes to store sperm, and total sperm mass may be constrained by the need for the male to remain sufficiently light to efficiently participate in nuptial flights.

Two additional variables can consistently explain lower but significant proportions of the remaining variation in sperm length: effective queen mating frequency (primarily in its interaction with other variables) and relative spermatheca size, with the interaction between them appearing in the simplest models in the 95% confidence set. The appearance of interaction terms between effective queen mating frequency and other predictor variables in most of the models in the 95% confidence set means that the relationship between these variables and sperm length is different in the monandrous and polyandrous species, which suggests that sperm competition may have played a minor role in shaping sperm length. The lack of a correlation between relative spermatheca size and sperm length in monandrous species suggests that queen storage constraints are relatively unimportant for the evolution of sperm length in monandrous species (underlining that the decrease in sperm length in Figure 2 is most likely due to male production constraints), whereas we found a trend that sperm length reflects the relative size of the available sperm stores of the queen in polyandrous species. As far as we know, this is the first study to report consistent comparative effects of relative male size on sperm production and of relative queen size on sperm storage.

The eusocial Hymenoptera provide rather extreme cases of production and storage constraints of sperm because males cease spermatogenesis after eclosion and females have to store a lifetime supply of sperm early in life. However, sperm-storage constraints are likely to apply to nonsocial insects as well as sperm storage by females is widespread (Birkhead and Moller 1998; Simmons 2001) and sperm-storage organs will always limit storage volume. Females might therefore have higher fitness when they store shorter sperm so that larger numbers can be stored per volume of storage organ. This may be particularly important when additional matings are costly in terms of searching, predation, or parasitism (Sheldon 1993) or when copulation itself affects fitness (Baer and Schmid-Hempel 2001; Brown and Baer 2005), for example, when seminal fluid components are toxic for the female (Wolfner 1997) or delay remating (Chen et al. 1988, Baer et al. 2001). From the male's perspective, having more but shorter sperm stored inside the female should result in the fertilization of more eggs and thus in higher male fitness, provided shorter sperm does not lose out in competition with other ejaculates for being stored. Males with continuous sperm production may also have upper limits on the volume of their ejaculates that may constrain the amount of sperm that they are able to transfer per copulation, especially when ejaculate size trades off with morphological or physiological traits (e.g., complex elaborations of male genitalia, accessory gland secretions, or male stamina) that affect male–male competition or female choice (Baer and Boomsma 2004; Fry and Wilkinson 2004; Poiani 2006).

Because queens of *Atta* and *Acromyrmex* leaf-cutting ants mate multiply (Reichardt and Wheeler 1996; Fjerdingstad and Boomsma 1998, 2000; Villesen et al. 2002; Sumner, Hughes, et al. 2004; Baer and Boomsma 2006) sperm competition might be present. An earlier study in *Acromyrmex versicolor* showed that no more than 10% of the inseminated sperm is transferred to the spermatheca (Reichardt and Wheeler 1996), a scenario that also applies to most honeybees

(reviewed in Baer 2005). Our statistical analysis did show a minor effect of multiple mating by queens on sperm length, but this appears to be via an increase in sperm-storage constraints in multiply mated queens. Multiple queen mating does not appear, therefore, to have directly and consistently selected for longer sperm in *Atta* and *Acromyrmex* ants. The reason for this ambiguous effect of mate number may be that the sperm-storage process varies across the polyandrous leaf-cutting ants because ejaculates in polyandrous social insects are either mixed in the queen's bursa copulatrix before storage of a fraction of all sperm or transferred directly to the spermatheca.

A recent study showed that males ejaculate sperm directly into the spermatheca in 2 species of *Atta* leaf-cutting ants (Baer and Boomsma 2006), similar to observations reported from 2 species of dwarf honeybees where sperm is transferred to the spermatheca during or immediately after copulation (Koeniger et al. 1989; Koeniger N and Koeniger G 1991). A direct sperm transfer to the spermatheca precludes sperm competition for storage, whereas indirect transfer allows it. The 2 *Atta* species for which direct sperm transfer has been confirmed, *A. colombica* and *A. cephalotes*, have longer sperm than the remaining leaf-cutting ants (Figure 2), which questions the generality of the idea that longer sperm evolves as a consequence of sperm competition before storage. If, on the other hand, sperm competition has a consistent length-reducing effect on sperm (see Garcia-Gonzalez and Simmons 2007), our data would predict that *A. sexdens* should have sperm mixing in the female reproductive tract prior to storage.

Even when competition for sperm storage is absent, sperm competition could still occur during egg fertilization after storage as multiple sperm are normally used to fertilize each egg (Baer 2003). Our results can thus not completely rule out that some form of sperm competition has affected sperm length evolution in leaf-cutting ants, but these effects seem unlikely to be major selective forces.

There are several promising areas for future research of possible effects of sexual selection in eusocial Hymenoptera with multiply mated queens. Apart from empirical testing for the presence of sperm competition during egg fertilization, reproductive conflicts in eusocial insects may include moderate hostility between unrelated ejaculates (see e.g., Baer and Schmid-Hempel 2005; reviewed in Boomsma et al. 2005). A deeper understanding of the selective forces that determine the morphology and paternity success of sperm in eusocial Hymenoptera must now come from studies on the actual processes of sperm storage, sperm mixing, and sperm use, on the details of sperm transfer, and on the physiological and morphological requirements of sperm that enable long-term storage (e.g., Baer et al. 2006).

FUNDING

Swiss National Science Foundation (stipend for advanced scientists to B.B.); Danish Natural Science Research Council to J.J.B. and B.B.; Danish National Research Foundation to M.B.D., D.R.N., and J.J.B.; and US National Science Foundation (award DEB-0110073) to U.G.M.

We thank Barbara Baer-Imhoof, Anna Himler, and Bill Hughes for help in collecting males in the field and in the laboratory, the Smithsonian Tropical Research Institute in Panama for providing logistic help and facilities to work in Gamboa, and the Autoridad Nacional del Ambiente of Panama for issuing collection and export permits. We thank Sean Brady and Ted Schultz for sharing unpublished phylogenetic information.

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