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Heritability of sperm length in the bumblebee *Bombus terrestris*

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Abstract

Sperm length is highly variable, both between and within species, but the evolutionary significance of this variation is poorly understood. Sexual selection on sperm length requires a significant additive genetic variance, but few studies have actually measured this. Here we present the first estimates of narrow sense heritability of sperm length in a social insect, the bumblebee *Bombus terrestris*. In spite of a balanced and straightforward rearing design of colonies, and the possibility to replicate measurements of sperm within single males nested within colonies, the analysis proved to be complex. Several appropriate statistical models were derived, each depending on different assumptions. The heritability estimates obtained ranged from $h^2 = 0.197 \pm 0.091$ to $h^2 = 0.429 \pm 0.154$. All our estimates were substantially lower than previous estimates of sperm length heritability in non-social insects and vertebrates.

Introduction

Sperm morphology is highly variable (e.g. Presgraves, Baker & Wilkinson , 1999) suggesting that selection on sperm traits might occasionally be intense (Birkhead & Møller, 1998; Simmons, 2001; Till-Bottraud et al., 2005). Sperm length received special scientific attention as it is highly variable between males, both within and between species (Gage et al., 1998; Ward, 1998; Joly, Korol & Nevo, 2004). A general explanation for the evolutionary significance of sperm length variation is still lacking (Simmons et al., 2003), as positive, negative and zero correlations between sperm length (as a phenotypic trait) and fitness have been reported (see Snook, 2005 for a recent review). Longer sperm has been hypothesized to be of selective advantage during sperm competition if larger sperm reaches eggs or sperm storage sites faster than shorter sperm (Gomendio & Roldan, 1991). A number of studies have indeed found support for this idea by associating sperm length with the risk or intensity of sperm competition (Briskie, Montgomerie & Birkhead 1997; Morrow & Gage, 2000; Balshine et al., 2001; Oppliger et al., 2003), but other studies did not confirm this hypothesis (Hosken, 1997; Stockley et al., 1997; Gage & Freckleton, 2003; Gage & Morrow, 2003; Simmons et al., 2003). Alternatively, sperm morphology might be under selection for optimal performance within the female sexual tract, implying that females select sperm morphology (Dybas & Dybas, 1981; Briskie, Montgomerie & Birkhead 1997; Miller & Pitnick, 2002, Baer et al., 2003), but it remains unclear whether a single optimum sperm length can be expected when sperm competition is absent.

Most studies have concentrated on measuring interspecific variation in sperm length, whereas less

attention was given to variation in sperm length among conspecific males or among sperm of the same male. However, the few data available suggest that intraspecific sperm length variation is as common and widespread as interspecific sperm length variation (e.g. Gage, 1998; Baer et al., 2003).

The haplodiploid social insects are an interesting group to study sperm morphology, because variation in sperm traits can be investigated at different levels (i.e. between species, between conspecific colonies, between brothers and within males) simultaneously and without constraints of low sample size (Baer et al., 2003). Compared to other organisms, individual social insect males can be expected to have lower phenotypic variance in sperm length because (1) males are haploid and produce clonal sperm (Bourke & Franks, 1995; Baer, 2003), and (2) males produce sperm only once, and early in life (Baer, 2003). Sperm competition is often absent in social insects because the females (queens) of most species mate only once, with a single male early in adult life (Boomsma & Ratnieks, 1996; Strassmann, 2001). This might either select for a single optimum sperm length with only little variation, or alternatively for the expression of substantial genetic variation because there is no selection on sperm length. Empirical evidence is scarce but indicates that variation in sperm length is large and comparable to diploid organisms (Baer et al., 2003): Sperm length differs significantly between (closely related) species of bumblebees (Baer et al., 2003), honeybees (Baer, 2005) and leaf cutting ants (B. Baer & J.J. Boomsma, in prep). In bumblebees, sperm length is also known to differ consistently between conspecific colonies and even between male siblings (brothers) raised in the same colony (Baer et al., 2003). Furthermore, preliminary data suggest that sperm length is important for male mating success in *Bombus terrestris*, although longer sperm is not generally more successful. Depending on the origin (genotype) of a mating pair either longer or shorter sperm may be preferentially stored in a female's spermatheca (Baer et al., 2003). Consequently sperm length seems of importance for a male's reproductive success but, as in other organisms, we lack a detailed understanding for the evolution and the selective maintenance of sperm length variability.

To understand the evolutionary significance of sperm length variation, we need to know whether sperm length has an additive genetic basis, as additive genetic variation is a necessary prerequisite for selection to act on sperm length via differential male reproductive success. We will estimate additive genetic variation and concentrate upon heritability. Such measures of genetic variation allow (1) the ability of a population to respond to selection to be predicted and (2) the strength of the forces that maintain genetic variation for a given trait to be assessed (Houle, 1992). The present study estimates the narrow sense heritability of sperm length in the bumblebee *Bombus terrestris*, a species of social insect that normally has simple full-sib societies (offspring of a single queen mated to a single male, Schmid-Hempel & Schmid-Hempel, 2000), but where queens sometimes mate with two males (Sauter et al., 2001, Röseler, 1973). First we show that it is straightforward to calculate heritabilities of male traits in social insects because haplo-diploid sex determination allows such estimations at multiple levels without intensive breeding programs over several generations (see Material and methods). Second, we apply a number of appropriate statistical models to our experimental data and show that they consistently produce moderately high heritabilities, but also a large unexplained variance in spite of carefully controlled experimental conditions.

Materials and methods

Data collection

Queens of *B. terrestris* were collected in the surroundings of Zurich (Switzerland) in spring 2002. They were kept in climate chambers at standardized conditions (28°C and 60% r.h) where they eventually started a colony and produced offspring. Queens and colonies were fed *ad libitum* with pollen and sugar water throughout the experimental rearing. As soon as colonies produced males we removed between 4 and 7 of these as newly eclosed callows from each of the colonies and kept them as brother groups in plastic boxes ($13 \times 6 \times 7$ cm) while feeding them *ad libitum* with pollen and sugar water. Sampling of newly eclosed males continued once a week for 4 consecutive

weeks, resulting in a maximum of 28 individuals per colony. Since worker reproduction, i.e. workers successfully rearing their own haploid eggs into male sons, is absent in Swiss *B. terrestris* this early in colony development (F. Theile, M. Bretscher, P. Schmid-Hempel, in prep.), we assumed to have collected only queen-produced males. We used 14 queens, and measured five sperm from each of 285 offspring males. The decision to measure five sperm per male was based on a pilot experiment using 20 sperm per male, after which we decided that five would be sufficient, according to the procedure outlined in Sokal and Rohlf (1981, chapter 10).

Sperm length was measured using a standard technique as described in Baer et al. (2003). Males were killed seven days after their removal from the colonies and sperm of the right accessory testis was dissected, smeared over a microscope slide and air dried. Afterwards pictures from non-damaged sperm were taken using a digital camera (Leitz) connected to a differential interference contrast (DIC) microscope (Leitz). Sperm length (in pixels) was measured by analyzing the pictures with a public domain NIH Image program (available at http://rsb.info.nih.gov/nih-image/). Male size was estimated by measuring the radial cell of the right forewing (in mm), which is correlated with body size (Mueller & Schmid-Hempel, 1992). All measurements were performed by the same person but were earlier found to be repeatable, both between experimenters and within the same experimenter (unpublished data)

Genetic variances

Several earlier studies have applied quantitative genetic techniques to haplo-diploid social and non-social insects, but they have rarely specified their methods in great detail and they never had to deal with a trait such as sperm length that can be sampled repeatedly from the same individual (Oldroyd & Moran, 1983; Moritz, 1985; Margolies & Cox, 1993; Boomsma et al., 2003). We therefore provide a detailed overview of our statistical methods below, before applying them to our data set.

First, we derive the theoretical expectation for the additive genetic variance based upon genetic theory. Once we have derived an expression for the additive genetic variance over all males in the population, we relate this additive genetic variance to the variance between groups of brothers, that is, to the variance over queens in the mean value of their sons. Second, we detail how these additive genetic variances can be estimated using nested analysis of variance. The same nested analysis of variance yields an estimate of the variance that is due to environmental factors. Heritability estimates thus express the additive genetic variance relative to the total variance, both genetic and environmental. Third, we indicate how we estimated the standard deviation of the heritability estimates.

The theoretical expectation for additive genetic variance can be based upon a genetic one locus model. In social Hymenoptera, gene expression at a putative locus A will differ between haploid males and diploid females. Allele A_1 occurs in the population with frequency p, and allele A_2 with frequency $q=1-p$. If female genotype A_1A_1 has genotypic value $-2a$, genotype A_1A_2 genotypic value d and genotype A_2A_2 genotypic value $+2a$, we can find the expected change in genotypic value if an A_2 allele is substituted for an A_1 allele. This average effect of an allele substitution equals $\alpha = -2a + d(q-p)$ in females, and equals the slope of the regression line connecting genotypic value (y-axis) to number of A_2 alleles (x-axis). The variance in genotypic value explained by this regression line is the additive genetic variance $V_A = 2pq\alpha^2$, whereas the dominance variance $V_D = (2pqd)^2$ corresponds to the non-explained variance. If male genotype A_1 has genotypic value $-b$, and male genotype A_2 has genotypic value $+b$, the average effect of an allele substitution in males equals $\beta = -2b$. The additive genetic variance among haploid males is $V_A = pq\beta^2$, but no dominance variance exists (Falconer & Mackay, 1996). The total additive genetic variance is assumed to be the sum of the additive genetic variances over loci. The specific purpose of the present study was to estimate the additive genetic variance in males.

To estimate the genetic variance of a male trait in a haploid social insect one needs a series of mothers and their sons. The genetic variance estimated over many mothers using the trait mean of sons per mother equals $1/2V_A = 1/2pq\beta^2$, reflecting a relatedness of 1/2 between the mother and her sons. The average genetic variance among sons of one mother averaged over all mothers in the populations is likewise $1/2V_A = 1/2pq\beta^2$, irrespective of whether the mother mates with a single or with many males, because fathers are genetically not represented in

male offspring. Over many loci, the population-wide variance of the means of sons per mother is $1/2V_A = \sum_{\text{loci } i} 1/2pq\beta_i^2$, which is equal to the average variance of sons within mothers for additive loci. Recombination does not increase the additive genetic variance of traits of haploid males within diploid mothers.

Variance components in ANOVA

In a trait like sperm length, the phenotype of a male can be measured repeatedly on different sperm from the same ejaculate. The statistical model for quantitative genetics of sperm length is therefore (Sokal & Rohlf, 1981; Falconer & Mackay, 1996):

$$Y_{ijk} = \mu + \alpha_i + \beta(\alpha)_{ij} + \varepsilon_{ijk} \quad (1)$$

where Y_{ijk} is the measured sperm length, μ the overall mean sperm length, α_i the effect of colony, i.e. mother, i, $\beta(\alpha)_{ij}$ the effect of offspring male j within colony i, and ε_{ijk} the error per individual sperm k. The effect of colony i would include both environmental differences between colonies that would finally influence sperm length, and genetic differences between the single queens that started the colonies. The non-genetic colony effects would lead to an expected variance component V_{EC}, whereas the genetic colony effects would lead to an expected variance component $1/2V_A$ (see above and Table 1). The effect of offspring male j within colony i would include genetic differences between males and environmental differences between males due for instance to differences in rearing conditions between brother males. This effect would lead to a male error variance within a colony V_{ES} and to an average genetic variation between males within mothers $1/2V_A$ (Table 1). Estimating added variance components from a nested (hierarchical) ANOVA with colonies (= mothers) as groups, males (= sons) as subgroups and sperm lengths as replicas would give two independent estimates of $1/2V_A$, if the variance components due to common environment (V_{ES} and V_{EC}) were both zero (Table 1). A significant difference between these added variance components would therefore indicate that at least one of these environmental variance components V_{ES} and V_{EC} is higher than zero.

Colonies were sampled once every week for a total of 4 weeks (sampling date). A possible statistical model including the effect of sampling date is the two factor model:

$$Y_{ijkl} = \mu + \alpha_i + \gamma_j + \alpha\gamma_{ij} + \beta(\alpha)_{ik} + \beta(\gamma)_{jk} + \beta(\alpha\gamma)_{ijk} + \varepsilon_{ijkl} \quad (2)$$

where Y_{ijkl} is the measured sperm length, μ the overall mean sperm length, α_i the main effect of colony, γ_j the main effect of sampling date, $\alpha\gamma_{ij}$ the colony by sampling date interaction, $\beta(\alpha)_{ik}$ the male within colony effect, $\beta(\gamma)_{jk}$ the male within sampling date effect, $\beta(\alpha\gamma)_{ijk}$ the male within colony by sampling date interaction and ε_{ijkl} the error across individual sperm.

Apart from sperm length, male body size was measured to estimate the heritability of male size and of any direct and indirect correlation between male size and sperm length. Including male size as a

Table 1. Expected mean squares and genetic variance components for a balanced design in a haplo-diploid system

Level	Factor	Expected MS	Represents	Added variance
Group:	Colony (Queen)	$\sigma^2 + n\sigma_M^2 + nb\sigma_C^2$	$V_E + n(V_{ES} + 1/2V_A) + nb(V_{EC} + 1/2V_A)$	$V_{EC} + 1/2V_A$
Subgroup:	Male	$\sigma^2 + n\sigma_M^2$	$V_E + n(V_{ES} + 1/2V_A)$	$V_{ES} + 1/2V_A$
Individual:	Sperm	σ^2	V_E	V_E
Estimation of added variance components				
Added variance due to colony effect:			$\frac{MS_{Group} - MS_{Subgroup}}{nb} = \frac{(\sigma^2 + n\sigma_M^2 + nb\sigma_C^2) - (\sigma^2 + n\sigma_M^2)}{nb} = \sigma_C^2$	
Added variance represents components:			$\sigma_C^2 = (V_{EC} + 1/2V_A)$	
Added variance due to male effect:			$\frac{MS_{Subgroup} - MS_{Individual}}{n} = \frac{(\sigma^2 + n\sigma_M^2) - (\sigma^2)}{n} = \sigma_M^2$	
Added variance represents components			$\sigma_M^2 = (V_{ES} + 1/2V_A)$	

σ^2 = the error variance between sperm lengths from same male; σ_M^2 = the added variance due to males; σ_C^2 = the added variance due to colonies; V_E = the environmental variance; V_{ES} = the variance due to a common 'body' environment, i.e. among sperm within a single male; V_{EC} = the variance due to a common rearing environment, i.e. of sperm within a single colony; V_A = the additive genetic variance; n = the number of sperm, b = the number of colonies.

covariate in the analysis of sperm length can be legitimately done in several ways. 1. Sperm length from a specific colony and sampling date might be regressed on mean male size, 2. Sperm length within a colony might be regressed on individual male size, or 3. Mean sperm length across all sampling dates might be regressed on male body size. After controlling for the effect of male size, model (1) might be applied again, to see whether the added variances of colony or male within colony have changed.

In both models (1) and (2) the added variance over colonies σ^2_C (derived from variation in model parameterα_i) represents the variance of the mean sperm length of sons across mothers, and equals $1/2V_A$, or $V_{EC} + 1/2V_A$ if a non-genetic colony-level difference exists between males (Table 1). The added variance between males within colonies σ^2_M represents $V_{ES} + 1/2V_A$ (Table 1). In model 2, the variance across males within the colony-by-sampling interaction (derived from variation in $\beta(\alpha\gamma)_{ijk}$) is likewise equal to $V_{ES} + 1/2V_A$, provided there is no added variance component of males within sampling date.

All statistical tests have been carried out in SPSS version 10.0. The factors of colony, sampling date and male size were all considered as random. SPSS type I sums of squares were specified in the SPSS syntax, as this corresponds to the computation of added variance components in Sokal and Rohlf (1981). Hierarchical ANOVA's were manually programmed in the syntax.

Standard errors of variance components and heritability

Heritability can be estimated in two ways. The first estimation uses the between colony variance over males, and can be written as (see Table 1)

$$h^2 = \frac{2\sigma^2_C}{\sigma^2_C + \sigma^2_M + \sigma^2} = \frac{2(V_{EC} + 1/2V_A)}{V_A + V_{EC} + V_{ES} + V_E} \quad (3a)$$

The second estimation uses the within colony variance between males, and can be written as (see Table 1):

$$h^2 = \frac{2\sigma^2_M}{\sigma^2_C + \sigma^2_M + \sigma^2} = \frac{2(V_{ES} + 1/2V_A)}{V_A + V_{EC} + V_{ES} + V_E} \quad (3b)$$

The denominators are identical, and the two numerators are two independent estimates of the same additive genetic variance if the environmental variances specific to colonies and to males within colonies are both zero.

The estimated variance components σ^2_C, σ^2_M and σ^2 all have their own sampling variance. The sampling variances of the estimated added variance components σ^2_C and σ^2_M can be obtained from Becker (1984) and Searle, Casella and McCilloch (1992), whereas the variance of the sample variance σ^2 of normally distributed measurements can be inferred from the chi-square distribution (Hays, 1988; Searle, Casella & McCilloch, 1992). The variance of the sample variance s^2 equals $\text{var}(s^2) = 2\sigma^4/(n-1)$. In our case, the parametric variance σ^2 is not known, so that we used the estimated error variance value V_E instead of the parametric variance that the formulas actually require (Searle, Casella & McCilloch, 1992). The variances and covariances of the added variance components corresponding to model (1) are given by Searle,Casella and McCilloch (1992; p. 430), under a similar substitution of the parametric variances by their estimates. The variance of the heritability is found by applying the expression for the variance of a quotient (Becker, 1984), and requires all added variances and their covariances. The derivation of the variance of heritabilities for model (2) was not attempted.

Results

Male body size

A total of 285 males from 14 colonies were available for statistical analysis. In a two factor ANOVA, male body size differed significantly between colonies ($p = 0.001$, Table 2) but was only suggestively different for sampling date ($p = 0.062$). However, the colony by sampling date interaction term for male size was also highly significant ($p < 0.001$), indicating that size differences among colonies varied considerably across sampling dates (Table 2). Heritability of male size can be estimated as twice the added variance percentage over colonies, where the added variance represents $V_{EC} + 1/2V_A$, leading to a heritability estimate for male size of $h^2 = 0.54 \pm 0.146$.

Table 2. A two factor ANOVA of male size, using colony and sampling as random factors

Factor	df	F	p	Added variance	% of variance
Colony	13	3.945	0.001	745.38	26.82
Sampling	3	2.678	0.062	122.38	4.40
Colony × sampling Interaction	36	4.132	<0.001	707.84	25.47
Error	232			1203.14	43.31

Total sample size was $N = 285$ males from 14 colonies. Sampling refers to sampling date when males where collected from the colonies.

When we ignored colony of origin or sampling date we found that larger males had longer sperm (correlation between male size and mean sperm length per male $r = 0.126$, $p = 0.033$, $N = 285$). However, within colonies the correlation between male size and mean male sperm length was significantly different from zero only in colony 163 ($r = 0.55$, $p = 0.007$) and high but not significant in colony 5 ($r = 0.44$, $p = 0.50$). On average the within colony correlations were similar to the overall correlation: $\bar{r} = 0.093$ (Figure 1). Per sampling date, the correlation between male size and mean sperm length in each colony varied between -0.21 and $+0.55$, with $\bar{r} = 0.058$. Mean male size per colony and mean male sperm length per colony were not correlated: $r = 0.001$, $p = 0.997$, $N = 14$.

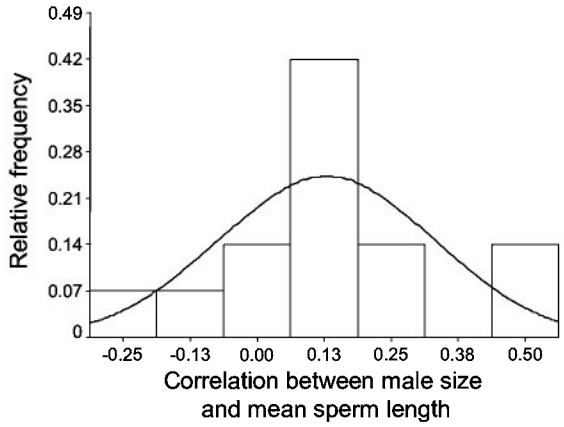

Figure 1. The relative distribution of correlation coefficients between male body size and mean sperm length per male across colonies ($N = 14$). The curve within the figure shows the predicted distribution of data under the assumption of normal distribution.

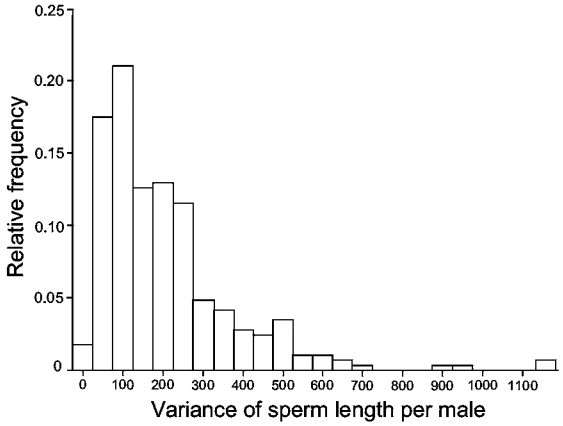

Figure 2. The relative distribution of the variances of sperm length within males ($N = 285$) across all experimental colonies. The mean variance of male sperm length was 214.448.

Sperm length variation within males

To test whether sperm length differs between sperm stored in the left and right accessory testis of a male (the organ where sperm becomes stored after maturation in Hymenoptera; Baer, 2003) we smeared 3–4 sperm subsamples of a male's left and right accessory testis and measured the length of 10 neighboring sperm on each slide. A total of six males from six different colonies were available. Sperm length differed significantly between males but not between the left and right accessory testis of a single male (nested ANOVA, between male: $F_{5,14} = 37.344$, $p < 0.001$, sperm samples within males $F_{14,180} = 0.663$, $p = 0.808$ n.s.). Sperm length was therefore considered to be homogeneously distributed within testes. Within males, sperm length was highly variable (Figure 2; Tables 3, 4 and 5) and the variance in sperm length was significantly negatively correlated with male size ($r = -0.118$, $p = 0.047$), indicating that small males had more variable sperm.

Measured sperm length over all males and error in sperm length within males were normally distributed (almost ideally for biological data).

Variance components of sperm length

The different available models gave slightly different estimates of the variance components. The

Table 3. Nested ANOVA statistics and added variance components explaining variation in sperm length according to model 1

Factor	df	F	p	Variance component estimate	% of variance
Colonies	13	5.269	<0.001	39.05	9.84
Males within colonies	271	4.335	<0.001	143.03	36.07
Sperm length within males	1140			214.45	54.08
Total				396.53	100

simplest model, a one factor ANOVA with mean sperm length per male as dependent variable and colony as factor yielded a highly significant difference between colonies (an added variance across colonies of 17.4% (i.e. 39.05/224.97). A two-factor ANOVA on mean sperm length per male with colony and sampling date as factors without nesting of data also yielded a highly significant difference between colonies ($F_{13,34.7} = 3.91, p = 0.001$) with an added variance across colonies of 16.0% (i.e. 35.88/224.86). Neither the effect of sampling date itself ($F_{3,349} = 0.876$, $p = 0.463$), nor the interaction term between colony and sampling week ($F_{36,232} = 1.141, p = 0.07$) was significant.

We further analyzed sperm length by a nested ANOVA according to model (1), although this implied that we had to ignore sampling date. Tests and variance components are given in Table 3 and show significant differences in sperm length both between colonies and between males within colonies. The variance component attributable to males within colonies is much higher than the variance component induced by the mean differences in sperm length between colonies (Table 3). This implies that, in terms of the quantities presented in Table 1, the variance component V_{ES} was certainly present, whereas the variance component V_{EC} might have been absent. A large part of the variation in sperm length between males must therefore be due to a factor that is independent of the colony that males live in, i.e. independent of their mother's genotype, but instead be due to individual differences between males. We proceeded to include the effect of sampling date and male size in the analysis, in the hope that either of these effects would remove the difference between the estimates of the added variance at the colony level and the male-within-colony level.

Analyzing the sampling dates separately showed that sperm lengths always significantly differed between males within colonies ($p < 0.001$). Differences in sperm length between colonies were found during the 1st, 2nd and 4th sampling but not for the 3rd sampling ($p = 0.002$, $p < 0.001$, $p = 0.183$, $p < 0.001$ for samplings 1–4, respectively). The variance components are given in Table 4.

The large variance of males within colonies (Table 3) and the difference between sampling dates (Table 4) implied that model 2 (the two factor ANOVA with males nested within colonies and sampling dates) might be a model to use (for results see Table 5). Colonies again differed significantly in sperm length ($p = 0.004$). The males-within-colony-by-sampling interaction was also highly significant ($p < 0.001$). The colony-by-sampling-date interaction and the males-within-colony factors were marginally significant. Sampling date and males-within-sampling date did not contribute to the variation in sperm length. Differences between sampling dates, that is, the age of the colony, did not contribute any variation in sperm length either. Thus, introducing sampling date in the model, as in model 2, did not remove the large difference between the variance components attributable to males within colonies and between colonies.

Male size was another possible candidate to contribute to the variation in sperm length, as larger males have on average longer and less variable sperm. However, using male size as a covariate to remove some variance had little effect on the added variance component across colonies (Table 6). The most pronounced, but still minor, effect of male body size is on the males-within-colony factor (model 1) or the males-within-colony-by-sampling-date factor (model 2).

Neither sampling date nor male size could explain the difference between the added variance at the colony level and the male-within-colony level. Neither model 2 nor including male size as a covariant constituted an appreciable improvement over model 1.

Heritability of sperm length

Sperm length in the experimental population varied due to the genotype of the mother queen, the

Table 4. Added variance components of sperm length using nested ANOVAs and repeating the analyses for the four sampling dates

Factor	Variance component estimates			
	Sampling 1	Sampling 2	Sampling 3	Sampling 4
Colonies	42.5(11.6%)	76.7(18.3%)	15.0(3.8%)	59.7(14.2%)
Males within colonies	75.7(20.8%)	136.1(32.5%)	174.0(44.6%)	163.2(38.9%)
Sperm length within males	246.2(67.6%)	206.0(49.2%)	201.4(51.6%)	197.0(46.9%)
Total	364.4(100%)	418.8(100%)	390.4(100%)	419.9(100%)

To facilitate comparisons between sampling dates, we have added the percentages of added variance in brackets.

genotype and rearing environment of the offspring males, and the error variance across individual sperm, whereas sampling date or male size had no consistent influence on the variance components of sperm length. Given this result, we can now use the added variance due to differences between colonies (i.e. mother queens; Table 3) ($\sigma^2_C = V_{EC} + 1/2V_A = 39$) and the added variance due to differences between males within colonies ($\sigma^2_M = V_{ES} + 1/2V_A = 143$), relative to the variance in sperm length within males ($\sigma^2 = V_E = 215$) and the total variance ($V_P = V_{EC} + 1/2V_A + V_{ES} + 1/2V_A + V_E = 39 + 143 + 215 = 397$), to estimate the heritability of sperm length (expression 3a, M&M). Clearly, the added variance between males within colonies is higher than the added variance between colonies (queens). We would have expected the opposite, as the queens that founded the colonies were collected from nature whereas males were raised under experimental conditions. Therefore, we expected the among-colony variance component to be potentially affected by several environmental factors that are unlikely to influence the among-brother component. However, given the outcome of the analysis, we conclude that the among-colony variance is the one most likely to represent the genetic variance in sperm length, and consider the estimate of half the additive genetic variance in males to be represented by $1/2V_A = 39$. If a component V_{EC} were present nevertheless, $V_A = 78$ would represent an upper boundary to the additive genetic variance. The unexplained environmental variance between males within colonies has a corresponding lower bound of $V_{ES} = 143 - 39 = 104$. Given the presence of this environmental variance, we do not use expression 3b to estimate heritability.

Table 5. Nested ANOVA statistics explaining variation in sperm length according to model 2

Factor 2		df	*F*	*p*
Colony	Hypothesis	13	3.059	0.004
	Error	37.6		
Sampling date	Hypothesis	3	1.145	0.369
	Error	12.6		
Colony × sampling	Hypothesis	36	1.531	0.040
date interaction	Error	160.5		
Male within Colony	Hypothesis	69	1.435	0.033
	Error	161.4		
Male within	Hypothesis	15	0.666	0.815
sampling date	Error	148		
Male within	Hypothesis	148	3.745	<0.001
colony × sampling	Error	1140		
date interaction				

A first approach to estimate heritability from these variance components is to use twice the between colony variance component divided by the total variance (expression 3a). This percentage represents the fraction of genetic variation of all individual sperm lengths. This heritability comes out at $2*39.05/396.53 = 0.197 \pm 0.092$ (using Table 3, see Becker, 1994 for the standard deviation of the estimate) and differs significantly from zero. Alternatively, two other estimates were considered, which both produced consistent results. The first assumes that the sampling dates represent independent estimates of the same proportion of heritable variation, and produced an average heritability of sperm length over the four sampling weeks of 0.24. The other used model 2 (data in Table 6, last column) and divided twice the added variance for the factor colony by the total variance to give an estimate of sperm length heritability of

2*35.88/396.42 = 0.181. The heritability estimates by the methods of Tables 3, 4 and 6 are thus consistent.

However, it is debatable whether this fraction of genetic variance and total variance is the most meaningful heritability. A bumblebee queen mates once, and at that event, an entire ejaculate of sperm from one male is transferred. It might be the mean sperm length of a male that is under selection, rather than the length of the individual sperm. If so, we have to use only the between colony component $V_{EC} + 1/2V_A = 39$ and the between male variance component $V_{ES} + 1/2V_A = 143$ when estimating the heritability of sperm length. In that case, the relevant total variance would be the sum of the between colony and the between males added variance components, leading to $h^2 = 2^* \ 39/(39 + 143) = 0.429$. This estimate is derived from the nested analysis of variance (Table 3) and therefore excludes any component derived from the within male variance in sperm length. The 95% confidence interval of the $h^2 = 0.429$ estimate was 0.149–0.753 (Sokal and Rohlf, 1989 box 9.3). This procedure to estimate heritability of sperm length, ignoring within-male variation, is the one found in the literature.

Using the rough method of direct ANOVA of colony differences in mean sperm length gave an added variance component between colonies of 39 and a within colony variance of 225, leading to a fraction genetic variation of $h^2 = 0.347$. The within colony variation now included a component derived from the variance in mean sperm length per male.

Discussion

Our different estimates consistently revealed significantly positive heritabilities for sperm length, which implies that sperm length in *B. terrestris* can be affected by sexual selection. This finding is consistent with the fact that bumblebee species with multiple mating (and thus supposedly sperm competition) have adaptively evolved longer sperm than other bumblebee species that have maintained single queen mating (Baer et al., 2003). However, it would also appear that significant sexual selection pressure would have the potential to erode genetic variation for sperm length, so that additive genetic variance for sperm length in *B. terrestris* is perhaps maintained merely because multiple mating of queens is rare. Below we will briefly evaluate the main results of our study: the technical difficulties in estimating heritability of sperm length, the comparative data on sperm length in other animals, and specific reasons why genetic variation for sperm length in haplodiploid social insects may remain low.

Our estimates of sperm length heritability in *B. terrestris* varied between 0.197 ± 0.092 and 0.429 ± 0.154 depending on the assumptions about the most relevant level of analysis. The numerator of these estimates was identical, but the proper denominator to choose remained ambiguous. The variance of sperm length within single males was fairly large (214.45, Table 3), leading to a standard deviation of 14.6 for a mean sperm length of 428.4 pixels (for length in μm see Table 7). As the genetic contribution to the variance among males within colonies was 39, the environmental variance V_{ES} among males within colonies could be estimated as $143 - 39 = 104$. The standard deviation of the mean sperm length within colonies, purely due to males and without any sampling variance across their sperm, therefore equaled 10.2. The observed actual length variation among clonal sperm in a single ejaculate was thus higher than the variation in mean sperm length across brother males within the same colony.

It came as a surprise that some unknown factor increased the variance component between brother males. The most obvious explanation for this effect would have been variation in body size, with larger males having longer sperm as has been reported previously for *B. terrestris* (Baer et al., 2003). However, this turned out to be only true for two colonies (5 and 163), whereas the overall correlation between sperm length and male body size remained low. Furthermore, including male body size as a covariate in the analysis did not remove any unexplained variation in the mean sperm length between males within colonies (Table 6). Our experimental setup controlled for nutrition, sampling date (male age), nest environment during rearing, and sperm length differences within the male sperm storage organs, so that none of these factors are likely to be the source of the unexplained sperm length variation across brothers This outcome differs from the results of a recent study in dung beetles where male condition (weight without the sexual organs corrected for

Table 6. Added variance components with male size included as a covariate in the models (1) and (2)

Model	Factor	Male size within colony/ sampling combination	Male size within colony	Male size over all data	Sperm length without male size
Model 1	Colony	40.47(10.96%)	39.65(10.30%)	40.15(10.21%)	39.05(9.85%)
	Male within colony	114.28(30.95%)	130.84(33.99%)	138.46(35.23%)	143.03(36.07%)
	Error	214.45(58.09%)	214.45(55.71%)	214.45(54.56%)	214.45(54.08%)
	Total	369.20(100%)	384.94(100%)	393.06(100%)	396.53(100%)
Model 2	Colony	33.30(9.02%)	34.56(8.98%)	34.50(8.78%)	35.88(9.05%)
	Sampling date	−0.29(−0.08%))	0.67(0.17%)	0.44(0.11%)	0.40(0.10%)
	Colony × sampling date interaction	22.25(6.04%)	11.10(2.88%)	12.50(3.18%)	12.74(3.21%)
	Male within colony	14.78(4.00%)	18.07(4.69%)	19.67(5.01%)	20.15(5.08%)
	Male within sampling date	−0.98(−0.27%)	−3.62(−0.94%)	−4.06(−1.03%)	−4.94(−1.25%)
	Male within colony × sampling date interaction	85.57(23.19%)	109.73(28.50%)	115.49(29.39%)	117.74(29.71%)
	Error	214.45(58.10%)	214.45(55.72%)	214.45(54.56%)	214.45(54.10%)
	Total	369.08(100%)	384.96(100%)	392.99(100%)	396.42(100%)

ANOVAS were repeated for the three different possibilities to enter the covariate in the models, either as a combination of colony and sampling (third column) date, as a covariate within colony (fourth column) or as a covariate over all data (fifth column). For comparison, model (1) and (2) were also run without male size as a covariate and results are given in the last column (see also Tables 3 and 5). Percentages of total variance explained are given in brackets to facilitate comparison between the different models used.

Table 7. Sperm length of the bumblebee *B. terrestris*, measured as an average of all sperm measured, using average sperm lengths per male, and average sperm length per colony

	N	Mean sperm length (μm)	SD	Range (μm)
Sperm length	1425	171.47	7.96	142.00–207.20
Sperm length between males	285	171.47	5.93	153.68–185.36
Sperm length between colonies	14	171.54	2.88	165.84–175.54

body size) appeared to affect sperm length, with males in better condition producing shorter sperm (Simmons & Kotiaho, 2002). However, this may be because there was much more opportunity for the expression of variation in individual condition in the dung beetle study than in our bumblebee experiment. The fact that our carefully controlled experimental procedure produced these surprising variance components underlines that our understanding of sperm length variation in bumblebees is still very incomplete.

Heritability estimates of sperm length are available for only a few other insect species but seem generally higher than our corresponding estimate of 0.429 in *B. terrestris*: 0.673 for the dung fly *Scatophaga stercoraria* (Ward, 2000), 0.52 ± 0.06 for the cricket *Gryllus bimaculatus* (Morrow & Gage, 2001) and 1.14 ± 0.61 for the dung beetle *Onthophagus taurus* (Simmons & Kotiaho, 2002). Each of these analyses used the mean sperm length per male as a quantitative trait, and thus a different procedure from the one we mainly used (and advocate), i.e. the one including the within male variation. Similar estimates for vertebrates gave even higher heritabilities for sperm traits. In rabbits sperm head length has been estimated to have a heritability of 0.72 ± 0.18 (Napier, 1961), whereas heritability of the sperm mid-piece length gave estimates of 0.97 ± 0.36 (Wooley & Beatty, 1967) and 0.76 ± 0.02 (Wooley, 1971) in mice. In a recent study of zebra finches, Birkhead et al. (2005) found heritabilities comparable to the upper confidence limits of our estimates for bumblebees (0.48 for sperm head length, 0.45 for sperm mid piece length, and 0.62 for sperm flagellum length). Although methods in these various studies differed (e.g. full sib analysis or parent–offspring analysis), all were comparable to our analysis that produced a heritability estimate of 0.429 (0.149–0.753) in *Bombus terrestris*. They also used mean sperm length per male and made no mention of within male variation in sperm length. Although it is important to realize that heritability estimates are valid only in the specific environment in which they were estimated (Hoffmann & Merila, 1999), it is perhaps remarkable that the overall trend in these comparative data seems to suggest that heritability of sperm traits may decrease with decreasing sperm competition.

The heritabilities reported in our present study are lower than the usual heritability estimates for life history traits (0.26 ± 0.01), physiological traits (0.33 ± 0.03) and behavioral traits (0.30 ± 0.02) which, in contrast to most morphological traits (0.46 ± 0.004), have a direct link to reproductive fitness (Mousseau & Roff, 1987). This would imply that the low but significant heritability of sperm length in *B. terrestris* remains puzzling. Evolvability (Houle, 1992) is also low: at an upper boundary estimate of the additive genetic variance of $2*39 = 78$ and a mean sperm length of 428.4 pixels, evolvability equals $\sqrt{78}/428.4 = 0.021$. Low heritability and low evolvability could imply past selection on sperm length. However, this seems not particularly likely as multiple mating seems an occasional and derived trait in *Bombus* bumblebees. One way to significantly advance our understanding of sperm length evolution may be to set up selection lines for sperm length, an approach that is feasible (albeit laborious) because an artificial insemination technique for *B. terrestris* has been developed (Baer & Schmid-Hempel, 2000).

An interesting novel development is that various insect studies have indicated that sperm length is determined by genes on the male specific chromosome in the dung fly *Scathophaga sterocoraria* (Ward & Hauschteck Jungen, 1993), the cricket *G. bimaculatus* (Morrow & Gage, 2001) and the dung beetle *Onthophagus taurus* (Simmons & Kotiaho, 2002), suggesting that such linkage might be a general rule. This result has been interpreted as evidence for a conflict over sperm length between the sexes: Females should be selected to pass on genes for shorter sperm because they might be easier or cheaper to store. Males on the other hand might be selected to produce longer

sperm if this is advantageous in sperm competition (Simmons & Kotiaho, 2002). An equivalent of this idea might apply in the eusocial Hymenoptera, where females may be selected to favor shorter sperm, when life time fertility requirements and sperm storage costs are high (Boomsma, Baer & Heinze, 2005). However, it is essential to note that the haplo-diploid social insects do neither have sex chromosomes, nor a paternal lineage as haploid fathers pass on their complete genome to daughters but have no sons. The absence of a male component in the hypothesized conflict over sperm length between the sexes might thus be an additional factor contributing to the relatively low heritabilities observed, but only when at least some sperm competition occurs.

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