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Research article

Rapid determination of sperm number in ant queens by flow cytometry

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Abstract. In social Hymenoptera, queens receive a given amount of sperm during a single or multiple inseminations once and for all. The amount of sperm stored at mating determines the maximum number of fertilized eggs queens can produce for the rest of their reproductive life. We propose flow cytometry (FCM) as a method to estimate the concentration of sperm cells, as well as their ploidy level, in queens' spermathecae. Our data, obtained from 5 ant species, show that FCM is precise, repeatable, easy to conduct and rapid. Estimates of variation of spermathecal content always remain below 10%, and samples can be analysed in less than 5 minutes. Flow cytometry appears as an excellent method for comparative analyses of sperm number within and between ant species.

Keywords: Sperm count, sperm cell ploidy, flow cytometry, ants.

Introduction

In most social insects, females mate only during a short period at the beginning of their reproductive lives and receive sperm for all later fertilizations. Thus, the upper limit of fertilized eggs queens can produce is directly dependent on the number of sperm stored in their spermatheca at the time of mating. Logically, precise determination of sperm cell concentration has become a current focus of studies on the evolution of mating systems in social insects. The content of queens' spermathecae has been examined for various Hymenoptera to investigate, e.g., the relationship between mating behaviour, sperm number, colony size and queen/colony fitness (yellow jackets: Stein and Fell, 1994; hornets: Stein et al., 1996; ants: Fjerdingstad and Keller, 2004; Reichardt and Wheeler, 1996; Wiernasz et al., 2001), the patterns of sperm transfer and sperm utilization (see review of Page, 1986; ants: Keller and Passera, 1992; Reichardt and Wheeler, 1996; Wiernasz et al., 2001), queen longevity and age from sperm depletion (ants: Tschinkel, 1987), sperm production and sperm competition (bumble bees: Duchateau and Mariën, 1995; honeybees: Moritz, 1986; Woyciechowski and Krol, 1996), or for tests of hypotheses on the evolution of multiple mating (e.g., "multiple-mating-for-more-sperm hypothesis"; ants: Fjerdingstad and Boomsma, 1998; Pearcy et al., submitted).

To date, estimation of the number of spermatozoa stored in the spermatheca has been studied according to two major procedures. A usual method consists in dissecting the spermatheca and dispersing sperm cells in a physiological buffer (usually saline) solution, and then counting samples of sperm suspension in aliquots placed in a cell count chamber (e.g., hemacytometers, Makler chamber) under a microscope (Duchateau and Mariën, 1995; Fjerdingstad and Boomsma, 1997, 1998; Fjerdingstad and Keller, 2004; Keller and Passera, 1992; Stein et al., 1996; Tschinkel 1987; Woyciechowski and Krol, 1996). The method can be refined by marking sperm cells with a fluorescent DNA-staining solution (e.g., Hoechst; Sakaluk and O'Day, 1984) and counting under a microscope equipped for epifluorescence microscopy, eventually after fixation in glacial acetic acid (Wiernasz et al., 2001). The total number of sperm cells in the sample is obtained by multiplying the average of several counts by the appropriate dilution factor. According to fluorometric sperm quantification (Reichardt and Wheeler, 1995), fixed sperm samples are sonicated, marked with a fluorescent DNA stain (e.g., Hoechst; Reichardt and Wheeler, 1995, 1996; Sakaluk, 1984), incubated for a few hours, and the fluorescence emitted by sperm heads is measured with a fluoroscan. The intensity of fluorescence is a linear function of the number of sperm counted in the sample.

However, both procedures have major drawbacks. Counting sperm cells through a microscope is tedious and time-consuming. The method is not highly precise; because of inherent errors in the technique, differences of up to 20 % are common between duplicate sperm count determinations (Freund and Carol, 1964). Moreover, the total number of sperm stored by each queen is roughly estimated by multiplying the average of several counts by a dilution factor. Finally, samples must be analysed rapidly after dissection, which may greatly limit the amount of data that can be accumulated at the time of mating. Fluorometric sperm quantification is reliable and allows processing larger number of samples than microscopy. But it takes time for incubation and, more importantly, a standard curve has to be established for every species. Furthermore, since data are based on fluorescence intensity only, they may potentially be biased by storage of diploid sperm cells if queens mated with diploid males.

Here, we propose the use of flow cytometry (FCM), a method for characterization of nuclear DNA variations (Aron et al., 2005; Boeck, 2001; Kron et al., 2007), to determine the number of spermatozoa stored in queen spermathecae from different species of ants. We show that FCM is a precise, repeatable and technically straightforward method of counting sperm. Additional advantages over existing techniques are that this approach is much faster than microscopy and fluorometry, and that it allows discriminating between haploid and diploid sperm cells.

Methods

Fifty queens from 5 ant species (10 per species) representative of 3 subfamilies were taken from field or laboratory-reared colonies: *Linepithema humile* and *Tapinoma erraticum* (Dolichoderinae), *Crematogaster scutellaris* (Myrmicinae), *Plagiolepis pygmaea* and *Lasius niger* (Formicidae). They were kept alive, except for *T. erraticum* whose queens were stored at − 80 °C for several months prior to dissection for other purposes. Queens of all species were of unknown age, and their mating frequency was not determined. Nevertheless, genetic analyses previously showed that queens of *L. humile* mate once (monandry) (Krieger and Keller, 2000), while queens of *L. niger* (Boomsma and Van der Have, 1998; Fjerdingstad and Keller, 2004) and *P. pygmaea* (Trontti et al., 2007) can mate multiply (polyandry). No information is currently available on queen mating frequency for *Tapinoma erraticum* and *Crematogaster scutellaris*.

The spermathecae were dissected out in deionized (Milli-Q) water and torn from the genital apparatus. They were placed in a drop of a ready-to-use solution of DAPI (4,6-diamidino-2-phenylindole; *CyStain UV Ploidy*, PARTEC©) for the fluorescent staining of nuclear DNA, ruptured, and the maternal tissue was carefully removed. The content of the spermathecae was then transferred into a vial, and DAPI staining solution was added to bring the final volume to 1 ml. The suspension was vortexed for 30 sec; ten 100 µL aliquots were immediately taken, placed in test tubes, and their final volume brought to 1 ml with the DAPI solution. Each tube was vortexed for 30 sec, and the number of sperm cells in each of them was estimated by using a PA-I flow cytometer (PARTEC©, Partec Gmbh, Münster, Germany). The following optical arrangement was used: KG1 (heat protection filter), UG1 (UV transmitting excitation filter), dichroic mirror TK420, GG435 (longpass filter) and 50x numerical aperture 0.82 achromate objective. PA-I HBO (high pressure mercury lamp) has a peak at 366 nm corresponding to DAPI and excitation energy is 100W. Activation is based on peak fluorescence only. Sample pre-run time was set to 3 (as recommended by constructor).

Data were collected for the total volume from each test tube at a speed of 1–5 µL/sec. Determination of the number of sperm cells was performed by gated counts, i.e., number of fluorescent events measured and displayed in the corresponding histogram section. By using this method, we obtained for each queen 10 estimates of the sperm concentration each from 1/10 of the spermatheca. We calculated the coefficient of variation (*CV*) of queen's spermathecal content from the mean and the standard deviation of the ten counts. We also estimated the total content of each queen spermatheca by summing the number of sperm cells in the ten counts. Data were normally distributed (Kolmogorov-Smirnov goodness of fit test). We used a nested ANOVA with two hierarchical levels to analyse sperm content variations among queens (queen-effect) and species (species-effect). Multiple comparisons among pairs of means were achieved by using Tukey's test.

Results

Flow cytometric analyses allowed assignation of ploidy level of sperm cells stored in queen spermathecae without any ambiguity. Indeed, the amount of DNA in diploid sperm nuclei was about twice as much as that of haploid sperm nuclei (Fig. 1). Few signals appeared in the region lower than the peak, indicating that the amount of disrupted nuclei (i.e., sperm loss) and/or non-specific staining of other cell constituents was low.

Nested ANOVA revealed a significantly lower intra-queen variability compared to inter-queen variations (nested ANOVA, queen-effect, $F_{45,450} = 636.3$, $p < 0.001$). In line with this result, for each queen the magnitude of the sperm content variation for the 10 aliquots sampled was reasonably low, comprised between 0.01 and 0.12 among all the queens sampled (Fig. 2). The mean coefficient of variation of queen's spermathecal content *CV* (± S.E. across queens; $n = 10$ queens sampled for each species) was 0.08 (± 0.008) for *T. erraticum*, 0.07 (± 0.003) for *P. pygmaea,* 0.05 (± 0.008) for *L. humile*, 0.03 (± 0.004) for *C. scutellaris*, and 0.03 (± 0.003) for *L. niger* (Fig. 2). Moreover, within each species sampled the mean number of sperm by queens greatly varied, and ranged from a ratio of 1 : 1.7 in *Crematogaster* to 1 : 5.5 in *Linepithema.*

Sperm content also significantly varied among species (nested ANOVA, species-effect, $F_{4,450} = 118.2$, $p < 0.001$), with mean number of sperm $X \pm SD = 59{,}020 \pm 24{,}960$ in *P. pygmaea;* $106{,}900 \pm 28{,}880$ in *T. erraticum*; $309{,}700 \pm 147{,}070$ in *L. humile*; $2{,}621{,}680 \pm 803{,}640$ in *L. niger* and $2{,}733{,}520 \pm 375{,}200$ in *C. scutellaris.* Such variations were closely associated with the social structure, with queens from monogynous species (*C. scutellaris*, *L. niger*) having significantly more sperm than queens from typically polygynous species (*P. pygmaea*, *L. humile*, *T. erraticum*) (Tukey test from nested ANOVA, $P < 0.001$ for all comparisons between monogynous *vs.* polygynous species).

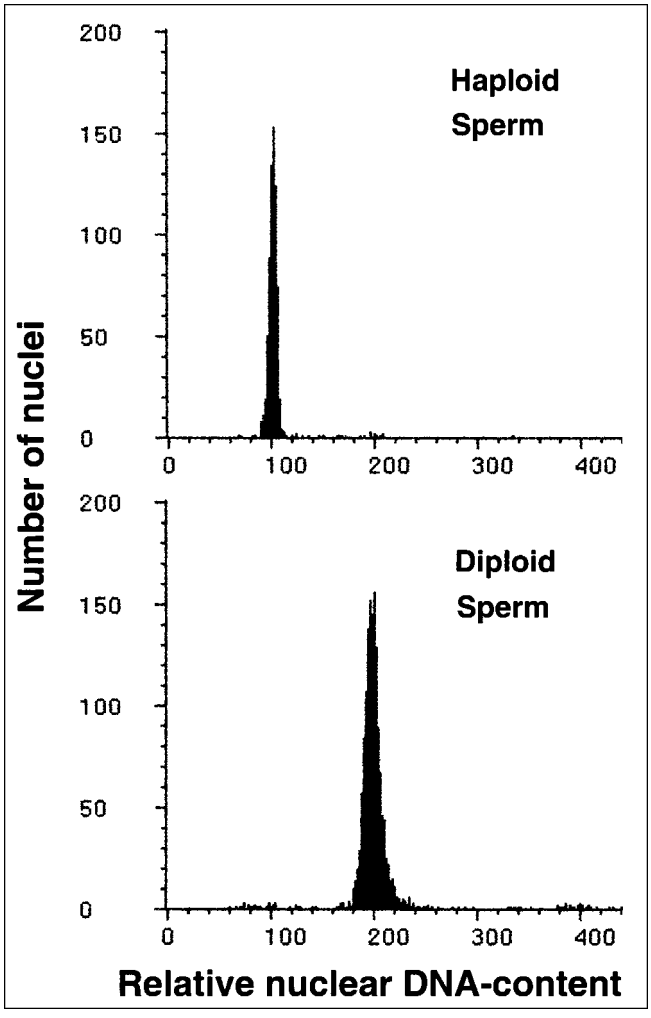

Figure 1. Flow-cytometric DNA-content histograms of haploid and diploid sperm from the spermathecae of two *Tapinoma erraticum* queens. Nuclear DNA-content was stained with DAPI solution. The flow cytometer was calibrated so that nuclei population from haploid sperm (queen 1, above) yielded a relative DNA content near channel 100, and nuclei population from diploid sperm (queen 2, below) yielded a relative DNA content near channel 200. Number of nuclei analysed was set so that peaks height reaches *ca*. 150 for both histograms.

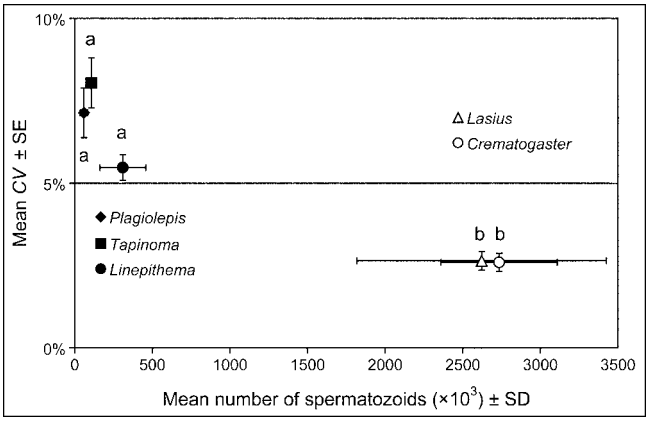

Figure 2. Mean coefficients of variation C*V* (± SE) of queen's spermathecal content in relation to the mean number of sperm cells (±SD) in the spermathecae for the five ant species sampled. Same letters indicate that mean sperm contents are not significantly different by Tukey test from nested ANOVA. Open symbols: monogynous species; filled symbols: polygynous species.

Discussion

The supply of sperm stored by queens of social insects at mating is crucial, because it is closely associated with the probability of successful nest founding, colony growth, productivity, and more generally colony fitness (Boomsma et al., 2005; Page, 1986; Stein et al., 1996). Our cross-species analysis indicates that FCM allows repeatable and fast estimation of the minimum number of spermatozoa stored in the spermatheca of ant queens. Despite the variation of 170–550 % in the number of sperm stored by queens, the coefficients of variation of spermathecal content from the 5 ant species studied remain remarkably low and always inferior to 10 %, which indicates high repeatability of the method. Therefore, future researchers using FCM with DAPI stain can either run the entire spermathecal content or only a sub-sample if they want to perform additional analyses (e.g., genetic analyses for paternity determination or queen mating frequency) on the same sample. Besides its repeatability, a major advantage of FCM is its rapidity. Once the spermatheca is dissected, labelling of its content with the fluorescent dye takes a few minutes (about 2–3 minutes per individual) and both the DNA-content of stained nuclei (i.e., ploidy level) and the number of sperm cells from each sample can be determined within a minute. Thus, time effort required to obtained results is greatly reduced as compared with other techniques, since it takes less than 5 minutes for determining sperm content of each female after dissection. Other studies have demonstrated that flow cytometry is the most precise and reliable technique (as compared to microscopy and fluorometry) for sperm counting in cattle (Evenson et al., 1993). Since the early 1980's, it has indeed been repeatedly used to count sperm production across mammals (Garner, 2001; Kron et al., 2007).

Counting methods are very sensitive to equal distribution of the sperm in the buffer; if sperm clumping occurs, the samples must be discarded. Flow cytometers working with a laser excitation source and, hence, DNA fluorescent stains such as acridine orange (AO) or propidium iodide (PI), allow determination of nuclear aggregates by pulse shape analysis, a process plotting fluorescence area *vs.* fluorescence width of incoming signals (Aron et al., 2003). Previous analyses performed on such cytometers showed that preparation of nuclear suspension results in very little artefacts (<2 %), such as fluorescent non-nuclear debris and nuclear aggregates when applied to sperm cells (Aron, unpubl. data). With HBO (high pressure mercury lamp), there is no way to perform pulse shape analyses for double events discrimination. In this situation, double counting issue is avoided by running nucleic acid analysis at low flow rates (50–100

nuclei/second, as recommended by current protocols in cytometry). Accordingly, the lack of significant fluorescent events (i.e., absence of peak) in channels of higher intensity than the main peak reported in our data (Fig. 1) shows that sperm clumping is rare or even absent. This strongly suggests that sperm collected from the female's spermathecae readily disperse in the DAPI solution.

Our data reveal large variations among queens from a same species in the minimum amount of sperm stored. These variations may stem from differences among queens in their age, mating frequencies, former productivity or even the amount of sperm received at the time of mating. Large differences in the amount of sperm stored by queens at mating were reported for various ants (e.g., *Atta colombica*, Fjerdingstad and Boomsma, 1998; *Acromyrmex versicolor*, Reichardt and Wheeler, 1996; *Linepithema humile*, Keller and Passera, 1992; *Lasius niger*, Fjerdingstadt and Keller, 2004). In *A. colombica*, some queens may store up to 33 times more sperm than others. Our data for the Argentine ant *L. humile* obtained by FCM can be compared with a previous study performed in this species by using microscopy (Keller and Passera, 1992). Queens of the Argentine ant are strictly monandrous (Krieger and Keller, 2000) and are short lived (Keller and Passera, 1989). Young, freshly mated queens collected from the field reportedly store 172,000 ± 76,000 (mean ± SE) sperm (Keller and Passera, 1992). Our data obtained by FCM from queens of more than 1 year-old show that they may actually accumulate on average 309,700 ± 46,510 (mean ± SE) sperm cells. We have no explanation to account for such a difference in the mean number of sperm in the spermathecae. Nevertheless, the much lower value of SE obtained by flow cytometry indicates that the method is much more precise than microscopy counting. Overall, we greatly encourage authors interested in the amount of sperm stored by queens (whether for intra- or inter-specific comparisons) to control for time of mating and queen mating frequency.

Interestingly, queens from monogynous species store more sperm than queens from polgynous ones. This could be due to high productivity of monogynous queens versus polygynous ones (Keller, 1988; Mercier et al., 1985; Vargo and Fletcher, 1989). However, both *L. niger* and *C. scutellaris* are characterized by relatively large colony sizes and high queen's lifespan. More likely, the amount of sperm stored by queens stems primarily on queen's lifespan and colony size, rather than social structure. One should therefore expect monogynous species with small size societies and/or high queen turn-over (i.e., short longevity) to store a lower amount of sperm. Consistent with this hypothesis, in the monogynous ant *Cataglyphis cursor*, a species with small colony size (Pearcy and Aron, 2006) and frequent queen replacement (Pearcy et al., 2006), queens store 109,700 ± 11,427 (mean ± SE) sperm cells (Pearcy et al., submitted), a value similar to that obtained in the polygynous species sampled in the present work. A possible association between the spermathecae content and colony size, queen reproductive longevity or social structure certainly merits further studies.

Finally, our results also show that flow cytometry may be successfully used for determination of ploidy level of sperm cells. Because of the complementary sex-determination (*csd*) system of Hymenoptera, homozygous individuals at the sex-determining loci develop into diploid males (Cook and Crozier, 1995). Diploid males constitute particularly high fitness costs to colonies since they are usually sterile or they father a sterile, triploid female progeny (de Boer et al., 2007; Cournault et al., 2006; Krieger et al., 1999; Liebert et al., 2004). Whether diploid males always produce diploid spermatozoa, or not, remains unclear; FCM could greatly help in this respect. More generally, flow cytometry should allow testing various hypotheses on the evolution of mating systems in social insects, including possible variations in male sperm production or in strategies of sperm utilization by females (e.g., Wiernasz et al., 2001; Schlüns et al., 2003).

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References

Aron S., De Menten L. and Van Bockstaele D. 2003. Brood sex ratio determination by flow cytometry in ants. *Mol. Ecol. Notes* **3:** 471–475

Aron S., de Menten L., Van Bockstaele D., Blank S. and Roisin Y. 2005. When hymenopteran males reinvented diploidy. *Curr. Biol.* **15:** 824–827

Boek G. 2001. Current status of flow cytometry in cell and molecular biology. *Int. Rev. Cytol.* **204:** 239–98

Boomsma J.J., Baer B. and Heinze J. 2005. The evolution of male traits in social insects. *Annu. Rev. Entomol.* **50:** 395–420

Boomsma J.J. and Van der Have T. 1998. Queen mating and paternity variation in the ant *Lasius niger. Mol. Ecol.* **7:** 1709–1718

Cook J.M. and Crozier R.H. 1995. Sex determination and population biology in the Hymenoptera. *Trends Ecol. Evol.* **10:** 281–286

Cournault L., Aron S. and de Biseau J.-C. 2006. Worker triploidy in a population of the erratic ant, *Tapinoma erraticum. Proc. 15th IUSSI Congr.*, p 158

de Boer J.G., Ode P.J., Vet L.E., Whitfield J.B. and Heimpel G.E. 2007. Diploid males sire triploid daughters and sons in the parasitoid wasp *Cotesia vestalis. Heredity* **99:** 288–94

Duchateau M.J. and Mariën J. 1995. Sexual biology of haploid and diploid males in the bumble bee *Bombus terrestris. Insect. Soc.* **42:** 255–266

Evenson D.P., Parks J.E., Kaproth M.T. and Jost L.K. 1993. Rapid determination on sperm cell concentration in bovine semen by flow cytometry. *J. Dairy Sci.* **76:** 86–94

Fjerdingstad E.J. and Boomsma J.J. 1997. Variation in size and sperm content of sexuals in the leafcutter ant *Atta colombica. Insect. Soc.* **44:** 209–218

Fjerdingstad E.J. and Boomsma J.J. 1998. Multiple mating increases the sperm stores of *Atta colombica* leafcutter ant queens. *Behav. Ecol. Sociobiol*. **42:** 257–261

Fjerdingstad E.J. and Keller L. 2004. Relationships between phenotype, mating behavior, and fitness of queens in the ant *Lasius niger*. *Evolution* **58:** 1056–1063

Freund M. and Carol B. 1964. Factors affecting haemocytometer counts of sperm concentration in human semen. *J. Reprod. Fertil.* **8:**149–55

Garner D.L. 2001. Sex-sorting mammalian sperm: concept to application in mammals. *J. Androl.* **22:** 519–526

Keller L. 1988. Evolutionary implications of polygyny in the Argentine ant, *Iridomyrmex humilis* (Mayr) (Hymenoptera: Formicidae): an experimental study. *Anim. Behav.* **36:** 159–165

Keller L. and Passera L. 1989. Size and fat content of gynes in relation to the mode of colony founding in ants (Hymenoptera; Formicidae). *Oecologia* **80:** 236–240

Keller L. and Passera L. 1992. Mating system, optimal number of matings, and sperm transfer in the Argentine Ant *Iridomyrmex humilis. Behav. Ecol. Sociobiol.* **31:** 359–366

Krieger M.J.B., Ross K.G, Chang C.W.Y. and Keller L. 1999. Frequency and origin of triploidy in the fire ant *Solenopsis invicta. Heredity* **82:** 142–150

Krieger M.J.B. and Keller L. 2000. Mating frequency and genetic structure of the Argentine ant *Linepithema humile. Mol. Ecol.* **9:** 119–126

Kron P., Suda J. and Husband B.C. 2007. Applications of flow cytometry to evolutionary and population biology. *Annu. Rev. Ecol. Evol. Syst.* **38:** 847–876

Liebert A.E., Johnson R.N., Switz G.T. and Starks P.T. 2004. Triploid females and diploid males: underreported phenomena in *Polistes* wasps? *Insect. Soc.* **51:** 205–211

Mercier B., Passera L. and Suzzoni J.P. 1985. Etude de la polygynie chez la fourmi *Plagiolepis pygmaea* Latr. (Hymenoptera : Formicidae). II. La fécondité des reines en condition expérimentale polygyne. *Insect. Soc.* **32** : 349–362

Page Jr R.E. 1986. Sperm utilization in social insects. *Annu. Rev. Entomol.* **31:** 297–320

Pearcy M. and Aron S. 2006. Local resource competition and sex ratio in the ant *Cataglyphis cursor. Behav. Ecol.* **17:** 569–574

Pearcy M., Hardy O., Aron S. 2006. Thelytokous parthenogenesis and its consequences on inbreeding in an ant. *Heredity* **96:** 377–382

Reichardt A.K. and Wheeler D.E. 1996. Multiple mating in the ant *Acromyrmex versicolor:* A case of female control. *Behav. Ecol. Sociobiol.* **38:** 219–225

Reichardt A.K. and Wheeler D.E. 1995. Estimation of sperm numbers in insects by fluorometry. *Insect. Soc.* **42:** 449–452

Sakaluk S.K. and O'Day D.H. 1984. Hoechst staining and quantification of sperm in the spermatophore and spermatheca of the decorated cricket, *Gryllodes supplicans* (Orthoptera: Gryllidae). *Can. Entomol.* **116:** 1585–1589

Schlüns H., Schlüns E.A, van Praagh J. and Moritz R.F.A. 2003. Sperm numbers in drone honeybees (*Apis mellifera*) depend on body size. *Apidologie* **34:** 577–584

Stein K.J. and Fell R.D. 1994. Correlation of queen sperm content with colony size in yellow jackets (Hymenoptera, Vespidae). *Environ. Entomol.* 23**:** 1497–1500

Stein K.J., Fell R.D. and Holtzman G.I. 1996. Sperm use dynamics of the baldfaced hornet (Hymenoptera: Vespidae). *Environ. Entomol.* **25:** 1365–1370

Trontti K., Thurin N., Sundström L. and Aron S. 2007. Mating for convenience or genetic diversity? Mating patterns in the polygynous ant *Plagiolepis pygmaea. Behav. Ecol.* **18:** 298–303

Tschinkel W.R. 1987. Fire ant queen longevity and age: estimation by sperm depletion. *Ann. Entomol. Soc. Am.* **80:** 263–266

Vargo E.L. and Fletcher D.J.C. 1989. On the relationship between queen number and fecundity in polygyne colonies of the fire ant, *Solenopsis invicta. Physiol. Entomol.* **14:** 223–232

Wiernasz D.C., Sater A.K., Abell A.J. and Cole B.J. 2001. Male size, sperm transfer, and colony fitness in the western harvester ant, *Pogonomyrmex occidentalis. Evolution* **55:** 324–329

Woyciechowski M. and Krol E. 1996. On intraviductal sperm competition in the honeybee (*Apis mellifera*). *Folia Biol. (Kraków)* **44:** 51–53