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Original article

Sperm numbers in drone honeybees (*Apis mellifera*) depend on body size

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Abstract – The effect of drone honeybee's body size on semen production was evaluated. In the same colonies, drones were either reared in drone cells (large drones) or in worker cells (small drones). Wing lengths (size indicator) and sperm numbers of small and large drones were compared. Small drones (~13% reduced wing size) produce significantly fewer spermatozoa (7.5 ± 0.5 million) than normally sized drones (11.9 ± 1.0 million spermatozoa). There is a significant positive correlation between sperm number and wing size within the small drones and in both groups combined. In the large group alone no correlation was found. The rearing investment per spermatozoon is lower for small than for normally sized drones because small drones produce more spermatozoa in relation to their body weight. Since colonies usually produce large drones, the enhanced investment must be outweighed by a mating advantage of large drones.

Apis mellifera / spermatozoa / male size / small drone / large drone / reproductive success

1. INTRODUCTION

In general, large males are considered to have a competitive advantage over smaller males when fighting for access to females (Thornhill and Alcock, 1983). However, small males have been shown to use specific tactics for gaining access to mating opportunities in a variety of species (Judson, 2002). In some solitary bees, male mating strategies vary greatly with body size (Michener, 2000). In social Hymenoptera only few genera have been reported to produce different male morphs. In the ant species *Formica exsecta* and *F. sanguinea* males of two distinct size categories have been described, which might be related to different dispersal strategies (Fortelius et al., 1987). In the communal halictine bee *Lasioglossum erythrurum* macrocephalic males fight within a nest and thus monopolize the

females therein (Kukuk and Schwarz, 1988). In the ant genus *Hypoponera* (Hamilton, 1979; Yamauchi et al., 2001) and in *Cardiocondyla obscurior* (Cremer et al., 2002) ergatoid males fight violently with each other. They have sabre-shaped mandibles and a massive head in contrast to the other male morph which is winged.

Male size also varies in the honeybee (*Apis mellifera* L.) but drones lack obvious combative traits or behaviours to compete for access to females. Indeed, the mating behaviour and the sequential copulations of many drones with one queen in flight within few seconds (Koeniger et al., 1979) seems to preclude any fighting among drones. However, even though there is no direct fighting among drones, body size might affect other traits concerning intra-sexual selection. Flight ability (Moritz, 1981)

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and semen production (Rinderer, 1985) have been shown to vary among drones. Both traits clearly affect their individual reproductive success. Furthermore, honeybee drones may compete *post copulam* since honeybee queens mate with numerous drones (Neumann and Moritz, 2000; Palmer et al., 2001; Franck et al., 2002). In contrast, drones mate only once in a lifetime since they die during the copulation process leaving a mating sign (secretions of male accessory glands) in the queen's sting chamber (Koeniger, 1990). The male monogamy is a peculiar character of honeybees distinguishing them from the closely related bumble bee (*Bombus terrestris*, Tasei et al., 1998) and many other insect mating systems (Thornhill and Alcock, 1983). After mating the semen of many drones is mixed (Haberl and Tautz, 1998) and stored in the queen's spermatheca, ensuring lifelong sperm usage for the fertilization of eggs (Page, 1986).

Combs in honeybee colonies are composed of two distinct types of cells. The worker caste is reared in smaller cells (5.2–5.8 mm diameter; Dietz, 1992), whereas the significantly larger cells (6.2 mm diameter) are used for rearing drones. The differences in body size among drones are mainly determined by this variation in brood cell size. Before a queen honeybee lays an egg into a cell, she evaluates cell size with her forelegs (Koeniger, 1970). Depending on cell width, she either lays an unfertilised male egg in a large cell or a fertilised female egg in a small cell. However, if the queen runs out of semen, she also lays unfertilised eggs in worker cells. Male larvae cannot develop into normally sized drones in these small cells. Small drones can also emerge when laying workers are present in the colony, although also workers preferentially lay eggs in drone cells (Page and Erickson, 1988).

On a drone congregation area in Germany the ratio of small to large drones was found to be about 1:10 (Berg, 1991). Thus, small drones appear frequently enough under natural conditions to play a substantial role in sexual competition among small and large drones. Body weights (reflecting investment of workers into the male) of small drones reared in worker cells are 41.9 to 52.3 percent lower compared to the weights of large drones reared

in drone cells (Gontarski, 1938; Berg, 1992). Thus, small drones are less costly and in addition no special drone combs have to be built for their rearing. However, honeybee colonies usually invest in large drones. The question arises: what are the benefits of large drone production for the colony? Using *cordovan* mutant honeybees Berg et al. (1997) showed that small drones reared in worker cells have a reproductive disadvantage compared to the normally sized drones. In spite of these differences, potential proximate mechanisms for the different reproductive success remained unidentified. Neither flight speed, flight height, nor sperm numbers per drone were significantly different between large and small drones in Berg and Koeniger's (1990) study. Berg et al. (1997) suggested that large drones might be more competitive in accessing the queen. However, in a subsequent study Jarolimek and Otis (2001) reported a significant correlation between drone weight and sperm number indicating that sperm number might nonetheless be an important factor for the reduced reproductive success of small drones. In light of this contradicting evidence and the potential impact on the evolution of the honeybee mating system, we here re-evaluate the effect of male body size on sperm numbers.

2. MATERIALS AND METHODS

2.1. Drone rearing

Small drones were produced in colonies which had exclusively worker cell combs. Ten virgin sister honeybee queens (*A. m. carnica*) were treated with CO₂ to initiate oviposition in late June 2002. All queens started laying drone eggs in worker cells. Combs containing all larval stages and pupae were transferred on the 23rd of July into eight equally sized large host colonies on the island of Neuwerk in the German Wadden Sea. All eight host colonies were also producing normally sized drones, in regular drone combs.

2.2. Sampling

“Large” and “small” drones were classified using a set of excluder grids (Fig. 1). Samples of large and small drones were taken on the 21st of August. The drones were transferred into a foster

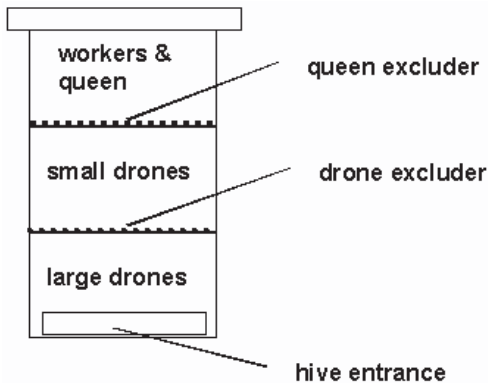


Figure 1. All drones and workers of the colonies were forced to re-enter their hives by first passing through a drone excluder (5.1 mm diameter, filtering large drones) and then a queen excluder (4.2 mm diameter) which cannot be passed by the smaller drones. The large drones could thus be sampled in the bottom super and the small drones in the centre super. The workers reached the uppermost super where the caged queen was located.

colony for a further four day sexual maturation period. Wing lengths, used as an indicator for drone size and weight (Berg, 1992), was determined by projecting the right forewing with a slide projector (50 times magnification, Hunt et al., 1998). Because wing tips are often torn, the distance between the two branching points *A* and *R* (Ruttner, 1988; p. 73) in the wing venation pattern was measured (precision ± 0.02 mm) rather than the total wing length. This distance reflects about 50% of the total wing length and is closely related to total body size (Ruttner, 1988).

The sperm numbers were counted by dissecting the *vesiculae seminales* of the drones. The tissue of one vesicle was torn to fine pieces with forceps in 0.5 mL Hyes' ringer solution (154.00 mmol/L NaCl, 2.68 mmol/L KCl, 1.80 mmol/L CaCl₂, 1.19 mmol/L NaHCO₃, pH 8.5; modified from Moritz, 1989). Distilled water was added to 5 mL total volume. The semen was mixed in the solution at both steps. Sperm numbers were counted under a phase contrast microscope by using a Thoma counting chamber in a total volume of 0.064 μ L. 16 replicate fields were counted and the average of the 16 spermatozoa counts was used as the best estimate spermatozoa count for that drone.

2.3. Statistical analysis

The differences in wing lengths between large and small drones was analyzed using a Student's

t-test. A Kolmogorov-Smirnov test was performed to check for a deviation from normality of the samples. In order to study potential differences in sperm numbers between large and small drones a two-level nested analysis of variance (ANOVA) was performed, taking into account the replicate measurements on each drone. A Pearson's correlation was calculated to test for an association between wing lengths and sperm numbers. All statistical analyses were computed using the STATISTICA™ software package (StatSoft, 2001).

3. RESULTS

Wing lengths and sperm numbers of 51 small drones and 32 large drones were measured.

3.1. Body sizes of drones

We found a mean wing length (distance A-R on the wing) of the large drones below the drone excluder of 6.03 ± 0.04 (s.e.) mm and 5.27 ± 0.03 mm for the small drones above (Student's *t*-test: $t = 16.2$, $df = 81$, $P < 0.001$).

3.2. Numbers of spermatozoa

The sperm numbers ranged from 1.094×10^6 to 30.312×10^6 spermatozoa. The overall mean was 9.187 ± 0.459 (s.e.) $\times 10^6$ spermatozoa per drone. The overall standard error of the measuring procedure (counting spermatozoa in the Thoma chamber 16 times) was $\pm 1.1 \times 10^6$ spermatozoa per drone. A mean sperm number of 11.948 ± 1.007 (s.e.) $\times 10^6$ was found for the large and a mean of $7.454 \pm 0.461 \times 10^6$ spermatozoa for the small drones. The Kolmogorov-Smirnov test did not reveal any departure from the normal distribution for both groups ($d_{\text{large}} = 0.17882$; $P > 0.2$; $d_{\text{small}} = 0.11642$; $P > 0.2$). The results of the nested ANOVA are summarized in Table I. The nested ANOVA yielded a highly significant difference between the sperm numbers in the large and small drones ($F = 20.76$, $df = 1$ and 81 , $P < 0.001$). The variance in sperm numbers among drones ($n = 83$) is significantly larger than the variance due to repeated measurements ($n = 16$) on each drone ($F = 13.415$, $df = 81$ and 1245 , $P < 0.001$).

Table I. Results of the two-level nested ANOVA: number of spermatozoa per counting field (16 fields per drone in the Thoma chamber) for 32 large and 51 small drones (** $P < 0.001$).

Source of variation	<i>df</i>	Sums of squares	Mean squares	<i>F</i>
among size groups (large and small drones)	1	1016.69	1016.69	278.529***
among drones within size groups	81	3966.28	48.97	13.415***
error; among counts	1245	4544.50	3.65	
total	1327	9527.47		

$F[1, 81] = 20.76^{***}$

3.3. Correlation between body size and number of spermatozoa

The correlation between sperm numbers and wing lengths is statistically highly significant (Fig. 2; $r = 0.487$; $P < 0.001$; $n = 83$; $df = 81$). It also remains significant even if the large drones are excluded from the analysis ($r = 0.406$; $P < 0.01$; $n_{small} = 51$; $df = 49$). However, there is no significant correlation within the group of large drones ($r = 0.06$; n.s.; $n_{large} = 32$; $df = 30$).

4. DISCUSSION

4.1. Variation of drone sizes

The wing length (distance A-R) of small drones, emerged from worker cells, is about 13% smaller compared to the wings of large drones which emerged from drone cells. This is substantially more than observed by Berg et al. (1997) who report on a 7% difference for wing length of small and large drones.

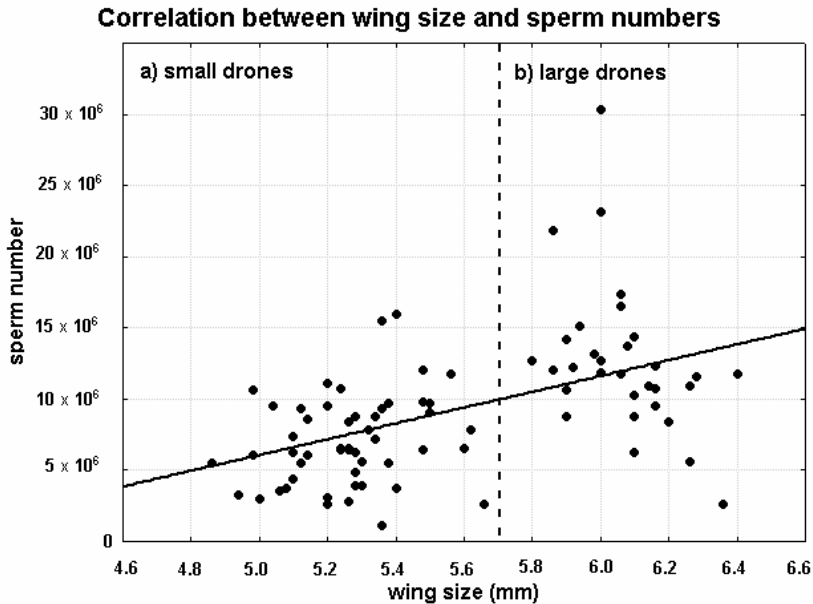


Figure 2. Correlation between wing size (measured as the distance A-R on the wing) and sperm number of small and large drones ($r = 0.49$; $P < 0.001$; $n = 83$): the small drones had wing measurements smaller than 5.7 mm.

This highly significant difference of wing lengths between small and large drones indicates that individual drones can be accurately assigned to either category just by wing size measurement.

4.2. Differences in sperm numbers among drones

The mean sperm number of 11.95×10^6 in the large drones are in good agreement with the findings of Woyke (1960; about 11×10^6 spermatozoa) in Poland, and with the study of Rinderer et al. (1985; 11.4×10^6 spermatozoa) on European drones in Venezuela. The sperm numbers are also within the same order of magnitude reported by Bassiouny (1992; 10.62×10^6 spermatozoa) on Carnolian drones. There are, however, remarkable variations among studies. For instance, Duay et al. (2002) counted noticeably lower sperm numbers (7.54×10^6 spermatozoa) for *A. m. carnica* in Tübingen, Germany. Moritz (1981) counted about 8.5×10^6 in Oberursel, Germany and Rinderer et al. (1999) 8.6×10^6 and 4×10^6 respectively in the same year. Berg and Koeniger (1990) found that large *A. m. carnica* drones had more spermatozoa (7.08×10^6) than small ones (6.76×10^6) albeit there was no significant difference between both groups in their study. Honeybees preferentially rear and foster large drones (Jarolimek and Otis, 2001). This might be one explanation (Berg, pers. comm.) for the difference between the previous study (Berg and Koeniger, 1990) and our findings. In our experimental set up large drones were competing with small drones during the larval rearing phase. This probably resulted in less favourable rearing conditions for the small drones. Another reason for the different findings might result from the breeding history of the drone mothers. Moritz (1981) found that drones of inbred queens produce fewer spermatozoa than drones of non-inbred queens. The age of drones could also play a role. Immediately after hatching there is no semen in the seminal vesicles and the numbers of spermatozoa only slowly increase (Bassiouny, 1992) until maturity is reached when drones are about two weeks old. However, since both studies were done with sexually mature drones this may not

have caused the discrepancy between the two studies.

4.3. A positive correlation of body size and sperm number

Our results confirm the significant positive correlation of body size and sperm number in honeybees reported by Jarolimek and Otis (2001). Both studies corroborate the potential importance of the differential semen production in small and large drones for male fitness in honeybees. The significant positive correlation of drone body size and sperm number is also supported by the fact that Africanized honeybees in South America have significantly lower body weights and at the same time lower sperm numbers than European drones (Rinderer et al., 1985).

4.4. Evolutionary perspective

Since in our study we find that small drones produce on average 37% less spermatozoa than large drones, large drones can be expected to be superior to small drones on the intrasexual selection level due to this difference. Indeed, also Berg et al. (1997) documented a reproductive disadvantage of small drones. However, the large and small drones, which were reared separately in their study, showed only minor differences in sperm numbers (Berg et al., 1997). Thus, other traits than sperm numbers additionally affect the reproductive competitiveness of the two distinct drone morphs.

Increased sperm numbers in larger drones only reflects the fitness benefit the colony could gain but not the costs of rearing. Discussing mating in *Pogonomyrmex* ants Hölldobler and Wilson (1990) state "... larger males mean fewer males per colony, an obvious trade-off in colony fitness ...". This should in general be the case in social insects. The workers in the honeybee colony determine the sizes and the frequencies of drones because they build the drone combs and rear the eggs to the adult stage. Thus, it seems plausible to assume that the production of large drones is adaptive at the colony level. By using our data on sperm numbers and data of Berg (1992) and Gontarski (1938) on body weights, we can roughly estimate the investment per

spermatozoon in both large and small male morphs. Large drones hold 45×10^6 spermatozoa per gram body mass, whereas small drones have 54×10^6 spermatozoa per gram body mass at the average. Small drones thus produce 20% more spermatozoa in relation to their body weight. This is surprising and one might expect a honeybee colony to gain more by producing many small drones rather than by a smaller, but equivalent number of large drones. Therefore, it seems safe to assume that traits other than sperm numbers have to outweigh the costly investment in large drones. The lower flight performance of small drones in terms of a lower daily flight duration (Berg, 1992) or a potential difference in semen quality could contribute to the preference to rear large rather than small drones. From our data we would predict a fitness advantage of at least twenty percent for the large drones to compensate for the more costly investment. A study quantitatively measuring the reproductive success of small and large drones might clarify the problem on the adaptive value of male size in honeybees.

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Résumé – Le nombre de spermatozoïdes dépend de la taille corporelle chez les mâles de l'Abeille domestique (*Apis mellifera*). Les mâles de l'Abeille domestique (*Apis mellifera* L.) sont élevés dans des cellules significativement plus grandes que les ouvrières. Si une reine est à cours de sperme, elle dépose des œufs de mâles dans des cellules d'ouvrières et ceux-ci donnent des petits mâles qui ont presque la taille des ouvrières. Berg et al. (1997) ont montré que les mâles de petite taille élevés dans des cellules d'ouvrières ont un désavantage reproductif lorsqu'ils sont en compétition avec des mâles de plus grande taille. Contrairement à Berg et al. (1990, 1997), Jarolimek et Otis (2001) ont trouvé une corrélation significative entre la taille des mâles et le nombre de spermatozoïdes. Nous avons réévalué l'influence de la taille corporelle sur la production de spermatozoïdes en raison de sa pertinence

pour l'évolution du système d'accouplement de l'Abeille.

Des reines vierges ont été traitées au gaz carbonique et ont pondu des œufs de mâles dans des cellules d'ouvrières. Les rayons correspondants ont été placés dans des ruches produisant des mâles de grande taille. On a prélevé des échantillons de mâles de petite et de grande taille en séparant les deux groupes par une grille à mâles. La longueur de l'aile a été prise comme indicateur de la taille corporelle (Berg, 1992). La mesure a été faite en agrandissant 50 fois l'aile antérieure à l'aide d'un projecteur et en mesurant la distance A-R (Ruttner, 1988) de la veination alaire. Les vésicules séminales des mâles ont été disséquées et les spermatozoïdes ont été comptés dans une chambre de comptage de Thoma sous microscope à contraste de phase. Les mâles de petite taille ont produit significativement moins de spermatozoïdes ($7,5 \pm 0,5$ millions) que les mâles de taille normale ($11,9 \pm 1,0$ millions). Les mâles de petite taille sont capables de passer à travers les grilles à mâles et se distinguent des mâles de plus grande taille par leurs ailes qui sont plus petites d'environ 13 %. La corrélation entre le nombre de spermatozoïdes et la longueur de l'aile est statistiquement hautement significative ($r = 0,487$; $P < 0,001$; $n = 83$). Elle reste également significative même si l'on exclut de l'analyse les mâles de grande taille. Ce résultat confirme l'importance potentielle de la différence dans la production de spermatozoïdes (Jarolimek et Otis, 2001) pour le succès reproductif des mâles de petite et de grande taille. Puisque les mâles de petite taille produisent relativement plus de spermatozoïdes par rapport à leur taille, l'investissement préférentiel des colonies d'abeilles dans des mâles coûteux doit être contrebalancé par des avantages compétitifs non identifiés des mâles de grande taille.

***Apis mellifera* / spermatozoïde / taille corporelle / succès reproductif**

Zusammenfassung – Die Körpergröße beeinflusst die Spermienanzahl bei Drohnen von Honigbienen (*Apis mellifera*). Drohnen von Honigbienen (*Apis mellifera* L.) werden in signifikant größeren Zellen aufgezogen als Arbeiterinnen. Wenn eine Königin keine Spermien mehr gespeichert hat, legt sie Drohneier in Arbeiterinnenzellen. In diesen Zellen können sich männliche Larven nicht zu normalen, großen Drohnen entwickeln. Berg et al. (1997) zeigten, dass kleine, in Arbeiterinnenzellen aufgezogene Drohnen in der Konkurrenz mit großen Drohnen einen Fortpflanzungsnachteil aufweisen. Jarolimek und Otis (2001) fanden – im Gegensatz zu Berg et al. (1990, 1997) – einen signifikanten Zusammenhang zwischen der Drohnengröße und der Spermienanzahl. Wir haben den Einfluss der Körpergröße auf die Spermienproduktion wegen seiner Bedeutung für die Evolution des Paarungssystems der Honigbienen

neu bestimmt. Jungfräuliche Königinnen wurden mit CO₂ behandelt und begannen Drohneier in Arbeiterinnenzellen zu legen. Die entsprechenden Waben wurden in große Drohnen produzierende Bienenstöcke platziert. Durch die Trennung beider Gruppen mittels eines Drohnenabsperrgitters konnten Stichproben von kleinen und großen Drohnen genommen werden. Die Flügellänge wurde als ein Indikator für die Größe von Drohnen genutzt (Berg, 1992). Für die Messungen wurden die rechten Vorderflügel mittels eines Diaprojektors auf das 50 fache vergrößert und die Distanz A-R (Ruttner, 1988) des Flügelgeäders gemessen. Die *vesiculae seminales* der Drohnen wurden präpariert und die Spermienzahlen mittels einer Thoma-Zählkammer und eines Phasenkontrastmikroskops bestimmt. Kleine Drohnen produzieren signifikant weniger Spermien ($7,5 \times 10^6$) als normal große Drohnen ($11,9 \times 10^6$). Die kleinen Drohnen können durch das Drohnenabsperrgitter gelangen und unterscheiden sich deutlich durch ihre kleineren (etwa 13 %) Flügellängen von den großen Drohnen. Der Zusammenhang zwischen der Spermienanzahl und der Flügellänge ist statistisch hoch signifikant ($r = 0,487$; $P < 0,001$; $N = 83$). Sie bleibt es auch, selbst wenn die großen Drohnen bei der Auswertung unberücksichtigt bleiben. Das Ergebnis bekräftigt die potentielle Bedeutung unterschiedlicher Spermienproduktion (Jarolimek und Otis, 2001) für den Fortpflanzungserfolg von kleinen und großen Drohnen bei Honigbienen. Da aber kleine Drohnen relativ mehr Spermatozoen im Verhältnis zu ihrem Körpergewicht produzieren, muss die bevorzugte Investition von Honigbienenkolonien in kostspielige Drohnen durch weitere bisher unbekannte Konkurrenzvorteile der großen Drohnen aufgewogen werden.

***Apis mellifera* / Spermien / Körpergröße / kleiner Drohn / großer Drohn / Fortpflanzungserfolg**

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