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Original article

Numbers of spermatozoa in queens and drones indicate multiple mating of queens in *Apis andreniformis* and *Apis dorsata*

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Summary — Drones of *Apis dorsata* had an average of $2.46 \cdot 10^6$ spermatozoa in their vesiculae seminales. Two queens had $3.67 \cdot 10^6$ spermatozoa in their spermathecae. In *A adreniformis*, drones had an average of $0.13 \cdot 10^6$ and the spermathecae of 2 queens contained 0.98 and $1.09 \cdot 10^6$ spermatozoa. In both *A dorsata* and *A andreniformis* the spermathecae of queens contained more spermatozoa than the vesiculae seminales of a single drone of either species. Therefore, we conclude that multiple mating occurs in both species as is the case for *A mellifera*, *A cerana* and *A florea*.

multiple mating / spermatozoon / *Apis andreniformis* / *Apis dorsata*

INTRODUCTION

In the past, sperm counts in drones and queens have provided valuable information on mating behavior. The high numbers of spermatozoa in the oviducts of freshly mated queens relative to the numbers in mature drones indicate that queens of *A mellifera* and *A cerana* are mated by an average of 8–10 drones on 1 mating flight (Tryasko, 1956; Woyke, 1960, 1975).

The numbers of spermatozoa in males and in spermathecae of queens have barely been studied in other species. Recently, in *A florea*, sperm counts of freshly mated queens revealed that, in this species, sperm is not injected into the oviducts but directly into the spermatheca. Drones only have about 1/3 as many spermatozoa in the vesiculae seminales as are contained in the spermatheca of the queen, suggesting that queens mate with 2–3 males (Koeniger *et al*, 1989).

In this paper we report numbers of spermatozoa of queens and drones of *A andreniformis* and *A dorsata*. Furthermore, the size of the spermathecae was

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measured and the number of ovarioles was counted.

MATERIAL AND METHODS

Samples of *A andreniformis* were collected in the Johore state, Malaysia. *A dorsata* drones were collected in the Selangor state, Malaysia and queens in the Anuradhapura district, Sri Lanka.

Drones were kept for some hours without food until they were too weak to empty the vesiculae seminales when killed by ether. Afterwards the drones were dissected and the vesiculae seminales were isolated. Drones were considered to be sexually mature when the testis had become degenerate and when the cornua had an orange color.

Queens were dissected and the spermatheca isolated. After removal of the tracheal net, the diameter of the globular spermatheca was measured under a microscope.

In each case, the organs were transferred into 1 ml 0.9% NaCl in order to count the spermatozoa. The organ was ruptured with fine forceps and the emerging spermatozoa were dispersed evenly in the fluid by heavily blowing air through it with a syringe. If the sperm concentration was high (opaque fluid), the sample was further diluted with up to 9 ml of distilled water.

A haemocytometer (Fuchs-Rosenthal or the "improved Neubauer") was used to count the sperm.

The fresh ovaries were isolated and divided into 4 portions; the number of ovarioles counted ranged between 10 to 30 per portion. Then 70% ethanol was poured over it; thus, the ovarioles became white and were easy to count.

RESULTS

Vesiculae seminales

In drones of *A andreniformis* (n = 5), the average number of spermatozoa in the vesiculae seminales was $0.13 \pm 0.01 \cdot 10^6$. In drones of *A dorsata* (n = 5) it was $2.46 \pm 0.15 \cdot 10^6$ spermatozoa (table I).

Spermatheca

All spermathecae were surrounded by a dense tracheal net and had a spermathecal gland similar in shape to that of *A mellifera*. The diameter of the spermathecae in both queens of *A andreniformis* was 0.8 mm. Because the spermathecal wall, as in all *Apis* species, is very thin, the volume of the spermatheca can be calculated directly from the diameter. It is 0.27 mm^3 in *A andreniformis*. The numbers of spermatozoa were 0.98 and $1.09 \cdot 10^6$. The diameter of the spermathecae of 2 *A dorsata* queens was 1.1 mm (volume: 0.7 mm^3). The sperm number was 3.69 and $3.64 \cdot 10^6$ (table II).

Ovarioles

Two queens of *A andreniformis* had 100 and 104 ovarioles (50 and 52 per ovary respectively), the 2 *A dorsata* queens had 230 and 236 ovarioles.

Table I. Sperm numbers of vesiculae seminales (x 10^6).
* (Koeniger *et al*, 1989)

A andreniformis	*A florea* *	*A dorsata*
0.12	0.45	2.24
0.13	0.46	2.56
0.14	0.40	2.46
0.12	0.39	2.44
0.13	0.48	2.64
	0.45	
	0.41	
$\bar{x} = 0.13$	$\bar{x} = 0.43$	$\bar{x} = 2.46$
$s = \pm 0.01$	$s = \pm 0.03$	$s = \pm 0.15$

Table II. Data of spermathecae of *A andreniformis*, *A florea*, *A dorsata*, *A mellifera* and *A cerana*. * (Koeniger *et al*, 1989), ** (Woyke, 1966), *** (Woyke, 1975).

	Diameter (mm)	*Volume (mm^3)*	*No sperm (10^6)*	*Concentr ($10^6/mm^3$)*
A a	0.8	0.27	1.09	4.03
	0.8	0.27	0.98	3.63
A d	1.1	0.70	3.64	5.20
	1.1	0.70	3.69	5.27
*A f**	0.8	0.27	1.35	5.00
	0.8	0.27	1.20	4.44
	0.8	0.27	1.20	4.44
	0.8	0.27	1.17	4.33
	0.75	0.22	0.85	3.86
*A m***	1.14	0.78	4.73	6.12
*A c****	0.98	0.48	1.35	2.79

DISCUSSION

In the species *A dorsata* and *A andreniformis*, the numbers of spermatozoa in the spermatheca exceeded those of the seminal vesicles of the respective drones. This verifies multiple mating for both species. Now multiple mating has been found in all known *Apis* species except *A koschevnikovi* where no data are yet available. Thus, polyandry appears to be a general feature of the *Apis* genus.

In *A mellifera* and *A cerana*, the number of matings cannot be deduced from the relation of the number of spermatozoa in the vesiculae seminales and in the spermatheca, because, in these species, drones inject the sperm into the oviducts while finally in the spermatheca less than 5% of the spermatozoa received during mating is stored.

In contrast to these results, no spermatozoa were found in the oviducts of freshly mated *A florea* queens; they were found only in the spermatheca (Koeniger *et al*, 1989). The endophallus does not end in a bulb as in *A mellifera* and *A cerana* but in a thin tip (Simpson, 1960; Ruttner, 1988), which seems to be an adaptation for direct sperm transfer into the spermatheca.

In *A andreniformis* the endophallus has a similar shape as in *A florea* and also ends in a thin tip (Wongsiri *et al*, 1990). Supposing that, in *A andreniformis*, the endophallus also enables direct sperm transfer, the number of matings would be 7–8. If the spermatozoa first reach the oviducts and the queen discards a lot of it, as in *A cerana* and *A mellifera*, a much higher number of matings can be assumed.

In *Apis dorsata* the size of the bulb is reduced and the cervix elongated compared to *A mellifera* and *A cerana* (Simpson, 1970; McEvoy and Underwood, 1988). There is no evidence to suggest that sperm is first injected into the oviducts or if a direct sperm transfer into the spermathe-

ca exists. In the case of direct sperm transfer, 2 drones are required to fill the spermatheca. If sperm transfer occurs as in *A cerana*, the number of matings would probably be much higher.

Sperm numbers in drones of *A florea* and *A andreniformis* are significantly different (table I; *t*-test, $P > 0.001$). This result also supports the view that these should be considered as different species.

The high number of ovarioles in queens of *A dorsata* suggests that she produces more eggs than the other 2 species (230 ovarioles compared with about 100). In queens from the Philippines, even more ovarioles per bee (260) were reported (Velthuis *et al,* 1971). Also the number of $3.7 \cdot 10^6$ spermatozoa, which is 3 times higher than in *A florea* and *A andreniformis*, supports this idea.

The concentrations of spermatozoa within the spermatheca appear to be similar in all *Apis* species, on average between 3 and $6 \cdot 10^6/mm^3$ (table II). The lowest value was found in *A cerana* (Woyke, 1975) with an average of $2.79 \cdot 10^6$ spermatozoa per 1 mm^3 (13 queens) and highest in *A mellifera* (Woyke, 1966) with $6.12 \cdot 10^6$ spermatozoa per 1 mm^3 on the average (126 queens).

The anatomy of the spermathecae is similar in all *Apis* species. They all are surrounded by a dense tracheal net and have a spermathecal gland as described for *A mellifera* by Snodgrass (1956). This may indicate that the mechanism of sperm storage and utilisation is similar in all species.

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Résumé — Le nombre de spermatozoïdes chez les reines et les mâles, preuve d'accouplement multiple des reines chez *Apis andreniformis* et *Apis dorsata*. Chez *Apis dorsata*, nous avons trouvé une moyenne de $2,46 \cdot 10^6$ spermatozoïdes dans les vésicules séminales des mâles et $3,67 \cdot 10^6$ de spermatozoïdes dans la spermathèque de 2 reines; chez les mâles d'*Apis andreniformis*, $0,13 \cdot 10^6$ de spermatozoïdes et chez les 2 reines examinées 0,98 et $1,09 \cdot 10^6$ (tableaux I et II). Le nombre de spermatozoïdes contenu dans la spermathèque des reines des 2 espèces est donc supérieur à celui produit par un seul mâle de chaque espèce. Ceci n'est possible que si les reines s'accouplent avec plusieurs mâles. Les reines d'*A dorsata* doivent s'accoupler avec au moins 2 mâles. Et si, lors de l'accouplement, comme c'est le cas chez *A mellifica* et *A cerana*, le sperme passe dans les oviductes et que seule une petite partie atteint ensuite la spermathèque, le nombre d'accouplement doit être sensiblement plus élevé.

Chez *A florea*, les spermatozoïdes passent directement dans la spermathèque. L'endophallus se termine par une pointe, et non par un bulbe, ce qui est considéré comme une adaptation au transfert direct (Koeniger *et al,* 1989). L'endophallus d'*A andreniformis* se termine également par une pointe (Wongsiri *et al,* 1990). C'est pourquoi nous admettons un transfert direct dans la spermathèque chez *A andreniformis* également. Dans ce cas les reines doivent s'accoupler avec 7–8 mâles. Nous avons donc une preuve de l'accouplement multiple des reines chez toutes les espèces récentes d'*Apis*, à l'exception d'*A koschevnikovi*, chez laquelle aucun comptage de spermatozoïdes n'a été effectué.

Les reines d'*A dorsata* renferment dans leur spermathèque presque 4 fois plus de spermatozoïdes que celles d'*A andreniformis*. Les concentrations par mm3 sont par contre semblables ($5,2 \cdot 10^6$ et $3,8 \cdot 10^6$, tableau II). La structure de la spermathèque est semblable chez toutes les espèces; c'est la taille qui diffère. Les spermathèques sont toutes entourées d'un réseau dense de trachées et possèdent toutes une glande spermathécale bifide (Snodgrass, 1956). Il en résulte vraisemblablement un stockage et une utilisation des spermatozoïdes pour la fécondation des œufs qui sont semblables chez toutes les espèces d'*Apis*.

Accouplement multiple / spermatozoïde / *Apis andreniformis* / *Apis dorsata*

Zusammenfassung — Die Anzahl Spermatozoen in Königinnen und Drohnen beweist eine Mehrfachpaarung der Königinnen bei *Apis andreniformis* und *Apis dorsata*. Bei Drohnen von *Apis dorsata* fanden wir im Durchschnitt $2,46 \cdot 10^6$ Spermatozoen in den Vesiculae seminales; zwei Königinnen hatten je $3,67 \cdot 10^6$ Spermatozoen in ihren Spermatheken. Bei Drohnen von *Apis andreniformis* wurden $0,13 \cdot 10^6$ Spermatozoen gezählt, bei den beiden Königinnen 0,98 und $1,09 \cdot 10^6$ (Tabelle I, und II). Die Spermatheken der beiden Honigbienenarten enthielten demnach mehr Spermatozoen, als ein einzelner Drohn der jeweiligen Art produziert. Das ist nur möglich, wenn sich die Königinnen mit mehreren Drohnen paaren. Die *A dorsata*-Königinnen müssen sich mit mindestens 2 Drohnen gepaart haben. Sollten jedoch die Spermien bei der Paarung wie bei *A mellifera* und *A cerana* zunächst in die Ovidukte übertragen werden, von denen nur ein kleiner Teil in die Spermatheka gelangt, muß die Anzahl der Paarungen wesentlich höher sein.

Bei *A florea* werden die Spermatozoen direkt in die Spermatheka übertragen. Der spitz auslaufende Bulbus des Endophallus wird als eine Anpassung an diese Funktion gedeutet (Koeniger *et al*, 1989). Der Endophallus von *A adreniformis* endet ebenfalls mit einer Spitze (Wongsiri *et al*, 1990). Deshalb nehmen wir auch bei *A andreniformis* eine direkte Spermaübertragung in die Spermatheka an. In diesem Fall sind die Königinnen von 7–8 Drohnen begattet worden.

Damit ist für alle rezenten *Apis* Arten – mit Ausnahme von *A koschevnikovi*, über die bisher keine Spermazählungen vorliegen – eine Mehrfachpaarung der Königinnen nachgewiesen. Die *A dorsata*-Königinnen enthalten fast viermal so viel Spermatozoen in den Spermatheken wie die Koniginnen von *A andreniformis*. Die Konzentrationen pro mm^3 dagegen sind ähnlich ($5,2 \cdot 10^6$ bzw $3,8 \cdot 10^6$, Tabelle II). Der Bau der Spermatheka differiert vor allem in der Größe, sonst sind sie ähnlich wie in *A mellifera* von einem dichten Tracheennetz umhüllt und haben eine zweiarmige Spermathekaldrüse (Snodgrass, 1956). Wahrscheinlich erfolgt die Speicherung der Spermatozoen und deren Aktivierung zur Eibefruchtung in allen *Apis* Arten nach einem ähnlichen Prinzip.

Mehrfachpaarung / Spermatozoon / *Apis andreniformis* / *Apis dorsata*

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