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Sperm utilization in honeybees (*Apis mellifera scutellata* and *A. m. capensis*) in South Africa*

Michael J. HOLMES¹, Michael H. ALLSOPP², Lee-Ann NOACH-PIENAAR³,
Theresa C. WOSSLER³, Benjamin P. OLDROYD¹, Madeleine BEEKMAN¹

¹ Behaviour and Genetics of Social Insects Lab, School of Biological Sciences A12, University of Sydney, NSW 2006, Australia

² Honeybee Research Section, ARC-Plant Protection Research Institute, Private Bag X5017, Stellenbosch 7599, South Africa

³ Department of Botany and Zoology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

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Abstract – We artificially inseminated queens of *Apis mellifera scutellata* and *A. m. capensis* with equal numbers of drones of both subspecies to determine the effects of sperm genotype on rates of sperm utilization. Contrary to a previous study we did not find a consistent overrepresentation of workers sired by *A. m. scutellata* males in the first four months after insemination. Interestingly, our study does suggest that there is a significant interaction between drone and queen genotype in both subspecies, with queens of each subspecies producing more workers sired by drones of the same subspecies.

Apis mellifera scutellata / *Apis mellifera capensis* / sperm competition / Africanization / hybrid zone

1. INTRODUCTION

South Africa is home to two subspecies of honeybee, the African bee *Apis mellifera scutellata* (hereafter *scutellata*), and the Cape honeybee, *A. m. capensis* (hereafter *capensis*). *Capensis* is confined to the southern part of the Western and Eastern Cape, whereas *scutellata* is found throughout the rest of South Africa and countries to its north (Hepburn and Radloff, 1998). The two subspecies interact within a hybrid zone, producing (when queens mate with males of both subspecies) mixed colonies comprising both pure and hybrid workers (reviewed in Beekman et al., 2008). *Capensis* is unique in that it is the only subspecies or species of *Apis* in which workers are capable of thelytokous parthenogenesis, the production of diploid offspring with-

out mating (Onions, 1912; Verma and Ruttner, 1983). This ability allows it to become a lethal social parasite of *scutellata* colonies, as *capensis* workers can enter a *scutellata* colony, activate their ovaries and lay eggs which in turn develop into reproductive adults (Martin et al., 2002; Beekman et al., 2008). Reproductive workers do not participate in normal hive duties, causing colony collapse if they become too numerous (Martin et al., 2002).

Scutellata was introduced into Brazil in 1956. Descendants of this introduction, known as the Africanized honeybee (AHB), have subsequently spread throughout the tropical and subtropical regions of the Americas (Spivak et al., 1991). The invasiveness of AHB in the Americas led to concerns that the *scutellata* population from which AHB descended would eventually overrun the *capensis* population (Anderson, 1980). Conversely, anthropogenic introductions of *capensis* into *scutellata*'s native range have led to the death of

Corresponding author: M. Beekman,
mbeekman@bio.usyd.edu.au

* Manuscript editor: Marina Meixner

thousands of *scutellata* colonies due to *capensis* parasitism (Allsopp, 1992). Yet, despite the competitive adaptations of both subspecies the hybrid zone separating *capensis* and *scutellata* appears to be stable (Hepburn and Crewe, 1991). It has been postulated that south of the zone, *capensis*'s social parasitism gives it a selective advantage, while in the north, *scutellata* has an advantage due to its high reproductive rate (Beekman et al., 2008).

In this study, we examined whether *scutellata* patrilines are over-represented in the offspring of both *capensis* and *scutellata* queens inseminated with equal numbers of drones of both subspecies. It was recently suggested that sperm of AHB males has an advantage over sperm from non-AHB males; when queens were artificially inseminated with sperm from both AHB and European drones, more workers were sired by AHB males compared with European males in months 2, 3 and 4 after inseminations (DeGrandi-Hoffman et al., 2003). If *scutellata* is able to out-compete *capensis* via sperm competition, then this may be one factor that gives *scutellata* a reproductive advantage in the northern part of the hybrid zone (Beekman et al., 2008).

2. MATERIALS AND METHODS

2.1. Queen-rearing

Capensis colonies used in this study were unselected colonies typical of those found around Stellenbosch, Western Cape (33° 56' S, 18° 51' E). *Scutellata* colonies were obtained from Douglas, Northern Cape (26° 01' S, 29° 22' E). Stellenbosch is well south of the hybrid zone whereas Douglas is well north of the zone within *scutellata*'s native range (Hepburn and Crewe, 1991). To reduce the likelihood of social parasites destroying our experimental colonies, the *scutellata* colonies were moved to an apiary separate from the *capensis* colonies near Stellenbosch. Queens were reared in late September and early October 2008. Queen-cells were harvested from the colonies nine days after grafting and emerged in an incubator at 35 °C. Upon emergence, the queens' wings were clipped and individually stored for genetic analysis. Newly emerged queens were placed with 20–30 newly emerged attendant bees until insemination.

2.2. Instrumental insemination of queens and collection of workers

Three *scutellata* and three *capensis* queens were artificially inseminated (Laidlaw, 1978) between five and nine days after emergence. For each queen we used semen from five *capensis* and five *scutellata* drones. Semen was collected alternately from drones of the two different subspecies. Once all semen was collected in the capillary, the queen was anaesthetised with CO₂ and inseminated. Even though we did not measure the exact volumes used, we took care that a similar amount of semen was used for each queen. We then introduced the queens into 5-frame *scutellata* colonies with *scutellata* workers and brood to maximise acceptance of the inseminated queens. As there are no diagnostic markers that distinguish *capensis* and *scutellata* subspecies (Franck et al., 2001), we kept the drones used for genetic analysis so that we could determine the father of the workers sampled.

As soon as queen-produced brood was about to eclose, we collected either pupae or freshly emerged workers from each colony. Thereafter we collected emerging brood at monthly intervals for three months. We collected approximately 100 workers from each of the six colonies at each sampling date. Previous findings suggest that if there is an effect of sperm competition it is apparent by the third month post insemination (DeGrandi-Hoffman et al., 2003). Therefore, sampling ended after the fourth month.

2.3. Genetic analyses

DNA was obtained from the queen (wingtips), the fathering drones and workers and pupae (2–3 legs) from each colony using a high salt extraction method (Aljanabi and Martinez, 1997). The fathering drones were screened with seven *Apis mellifera* microsatellite markers used in previous parentage studies: Am005, Am006, Am008, Am046, Am052, Am059 and Am061 (Salignac et al., 2003). For colony C1, one microsatellite marker was sufficient for distinguishing *capensis* and *scutellata* patrilines (Am061). For the other colonies, duplex polymerase chain reactions were required (Colonies S1 and C2: Am008/Am061, Colonies S2 and S3: Am008/Am059, Colony C3: Am046/Am061).

PCR product (0.4 µL) from each multiplex reaction was added to 10 µL formamide and 100 nL LIZ DNA size standard (Applied Biosystems). Samples

were run on a 3130xl Genetic Analyser (Applied Biosystems) with capillary length 36cm and injection time of 15 s at 1200 V for 41 min. Results were analysed using Genemapper 3.7 (Applied Biosystems) and the patriline (*capensis* or *scutellata*) of each individual was determined.

2.4. Statistical analyses

We calculated the proportion of workers sired by *scutellata* drones produced in each month by each queen. We used contingency tests on the number of workers sired by *capensis* and *scutellata* fathers to test for change in this proportion each month by each queen and to compare the total number of *scutellata*-patrilines produced by each queen subspecies. We further tested if colonies within subspecies show the same directional change using χ^2 -tests of heterogeneity.

3. RESULTS

We found a high degree of variability among the colonies in the number of workers sired by *scutellata* drones (Tab. I and Fig. 1). The number of *scutellata*-patriline individuals changed significantly over time in 3 of 6 colonies (Tab. I and Fig. 1). Sperm use within queen subspecies was variable (Tab. I and Fig. 1). There was a significant effect of queen genotype on the number of *scutellata* derived workers, with *scutellata* queens producing a significantly higher number of *scutellata* patriline workers than *capensis* queens (data pooled per queen subspecies, $\chi^2_1 = 114.593$, $P < 0.001$) (Fig. 1).

4. DISCUSSION

DeGrandi-Hoffman et al. (2003) found a significant increase in the number of workers sired by *scutellata* drones in the second, third and fourth month after insemination and concluded that this was evidence for sperm competition. Contrary to their results we found no directional increase in the contribution of *scutellata* drones. If anything, our results seem to suggest that the number of *scutellata*-produced offspring decreases over time,

Table I. χ^2 tests of the change in number of workers sired by *scutellata* drones over time (months 1–4, apart from colony S2 which lost its queen during the fourth month before samples could be collected) per colony (S1–S3: colonies headed by *scutellata* queens, C1–C3: colonies headed by *capensis* queens). See Figure 1 for a graphical representation of the data. A significant bias towards use of sperm of a particular subspecies was detected in colonies S3, C1 and C3 (see Fig. 1 for the direction of change). To determine if colonies within subspecies show the same directional change, we performed heterogeneity tests. Heterogeneity χ^2 were calculated by taking the absolute value of the difference of ' χ^2 of Total' and the 'Total of χ^2 '. p -values were calculated from the χ^2 -distribution of the χ^2 -values and degrees of freedom. Within queen genotypes there was a significant heterogeneity among colonies.

<i>Scutellata</i> queens			
	χ^2	df	P
S1	6.128	3	0.106
S2	2.969	2	0.227
S3	13.664	3	0.003
χ^2 of Total	10.535	3	0.015
Total of χ^2	22.761	8	0.004
Heterogeneity	12.226	5	0.032
<i>Capensis</i> queens			
	χ^2	df	P
C1	18.992	3	<0.001
C2	1.348	3	0.718
C3	15.323	3	0.002
χ^2 of Total	20.749	3	<0.001
Total of χ^2	35.663	9	<0.001
Heterogeneity	14.914	6	0.021

especially in *capensis* queens (Fig. 1). Although the number of spermatozoa produced by *capensis* (8.9 ± 1.1 million, Buys, 1990) and AHB (9.2 ± 1.8 million, Rinderer et al., 1985) drones are similar (no data are available for *scutellata* in South Africa), the variance among drones is enormous within subspecies and even breeder lines (Koeniger et al., 2005). Our colonies varied significantly in the number of *scutellata* workers produced over time, and this may possibly be explained by differences in spermatozoa numbers among drones. We note however, that the number of spermatozoa produced by drones is not

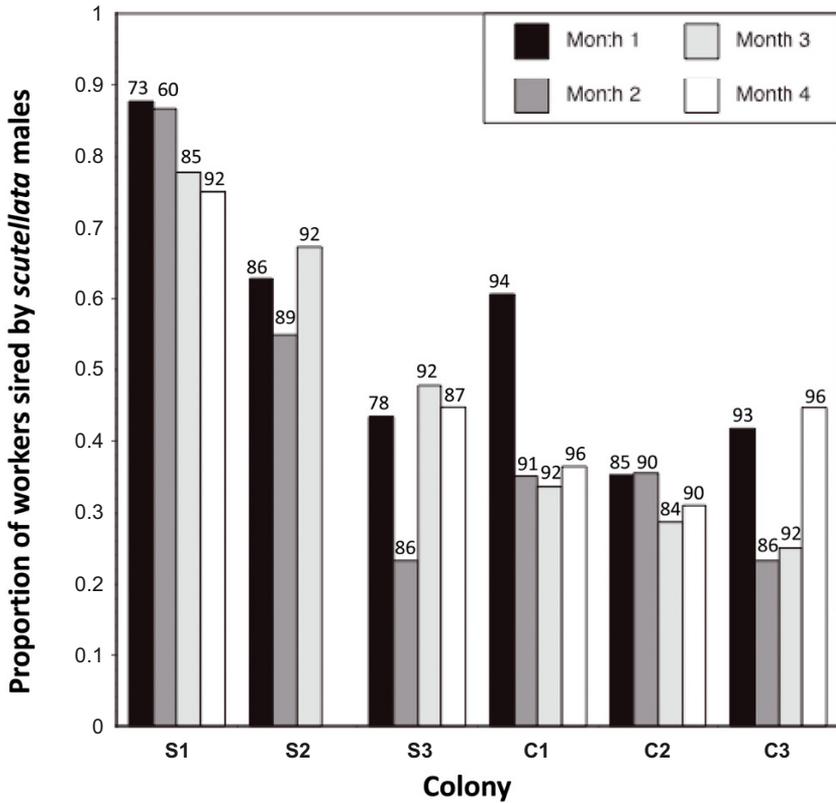


Figure 1. Proportion of individuals sired by *scutellata* drones produced in each month by *scutellata* (S1–S3) and *capensis* queens (C1–C3). A significant bias towards use of sperm of a particular genotype was detected in colonies S3, C1 and C3 (see Tab. I). Numbers above bars represent the total number of bees successfully genotyped for that sampling date. When pooled across queen genotype *scutellata* queens produced a significantly higher proportion of *scutellata* patriline workers than *capensis* queens ($\chi^2_1 = 114.593$, $P < 0.001$).

directly correlated with paternity frequency as spermatozoa numbers are significantly lower in AHB drones compared with European drones (Rinderer et al., 1985). Nonetheless in the study by DeGrandi-Hoffman et al. (2003) AHB drones sired disproportionately more workers than European drones.

Interestingly, our results also show that *capensis* queens produced more *capensis* patriline offspring, while *scutellata* queens produced more *scutellata* patriline offspring despite the presence of sperm of both subspecies in the queens' spermatheca (Fig. 1). Such an effect of queen subspecies was absent in the study of DeGrandi-Hoffman et al. (2003). Our results suggest that *scutellata* sperm may be

disadvantaged in *capensis* queens. A similar 'same subspecies advantage' has been reported in mating swarms of mixed honeybee subspecies where queens produced more offspring sired by drones of their own subspecies (Koeniger et al., 1989). The mechanisms that lead to assortative paternity are unknown, but in our study we can exclude any effect of female mate choice (Baer, 2005). Assortative paternity in our study could have arisen either via cryptic female choice prior to fertilization, or the preferential rearing of pure subspecies' offspring. The latter would lead to an increase over time of workers sired by drones of the same subspecies as the queen, consistent with our results when the data are pooled

across queen subspecies. However, given the small colony-level sample size and the significant variation among colonies within the same subspecies, we recommend that the conclusion that offspring of each subspecies are overrepresented in hybrid colonies headed by queens of the same subspecies be viewed with caution. A more thorough study is required. Should it be confirmed however, then this would be a significant factor in the *capensis-scutellata* hybrid zone dynamics and in the stability of the hybrid zone (Beekman et al., 2008).

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Utilisation du sperme chez les abeilles (*Apis mellifera scutellata* and *A. m. capensis*) en Afrique du Sud.

Apis mellifera scutellata / *Apis mellifera capensis* / compétition spermatique / africanisation/ zone hybride

Zusammenfassung – Spermiennutzung bei Honigbienen (*Apis mellifera scutellata* und *A. m. capensis*) in Südafrika. In Südafrika kommen zwei verschiedene Unterarten der Honigbiene vor: die Kaphonigbiene, *Apis mellifera capensis*, und die afrikanische Honigbiene, *A. m. scutellata*. Die beiden Unterarten sind durch eine Hybridisierungszone getrennt, in welcher sie sich miteinander kreuzen. Die Kaphonigbiene ist als sozialer Parasit der afrikanischen Honigbiene bekannt und hat massive Völkerverluste verursacht, als sie in Gebiete eingeführt wurde, wo normalerweise nur *A. m. scutellata* vorkommt. Interessanterweise scheint *A. m. capensis* trotz ihrer Fähigkeit zum Parasitentum nicht in der Lage zu sein, die Hybridisierungszone ohne Hilfe zu durchqueren. Auch *A. m. scutellata* hat besondere Eigenschaften. Nach ihrer Einfuhr nach Brasilien im Jahr 1956 hat sie im Laufe von 20 Jahren alle anderen dort vorkommenden Unterarten der Honigbiene durch Konkurrenz verdrängt. Ihr Erfolgs-

geheimnis scheint dabei ihre besonders hohe Vermehrungsrate zu sein. In einer kürzlich veröffentlichten Studie wurde vorgeschlagen, dass *A. m. scutellata* auch durch Spermienkonkurrenz in der Lage ist, andere Unterarten auf den amerikanischen Doppelkontinent zu verdrängen. Hier haben wir untersucht, ob es in Königinnen, die sowohl mit Sperma von *A. m. capensis* als auch von *A. m. scutellata* besamt wurden, zu Spermienkonkurrenz kommt. Wir haben sowohl Königinnen von *A. m. capensis* als auch von *A. m. scutellata* mit Sperma, das von einer gleichen Anzahl Drohnen beider Unterarten abgenommen wurde, besamt. Durch monatliches Ab sammeln und Genotypisieren von Arbeiterinnen-Nachkommen haben wir den relativen Anteil der *A. m. scutellata* Drohnen an den erzeugten Arbeiterinnen bestimmt. Wir haben keinen höheren Anteil von Arbeiterinnen mit *A. m. scutellata* Vätern gefunden. Unsere Ergebnisse scheinen höchstens anzudeuten, dass Königinnen das Sperma von Männchen ihrer eigenen Unterart bevorzugen. Ob dieser Effekt wirklich besteht, muss in einer ausführlicheren Untersuchung geklärt werden.

Apis mellifera scutellata / *Apis mellifera capensis* / Spermienkonkurrenz / Afrikanisierung / Hybridisierungszone

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