Apidologie 41 (2010) 129–134
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DOI: 10.1051/apido/2009054

Original article

Clinal nature of the frequencies of ovarioles and spermathecae in Cape worker honeybees, *Apis mellifera capensis**

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Received 29 January 2009 – Revised 29 April 2009 – Accepted 22 May 2009

Abstract – It was determined that 300 Cape workers, *Apis mellifera capensis* (collected from each of 6 colonies at each of 5 localities about 200 km apart along an 800 km transect in the Western and Eastern Cape Provinces, South Africa) was the sample size required to statistically estimate the proportions of workers with spermathecae at each location at 95% confidence levels. Because of the extremely clinal nature of this honeybee population, we tested the hypotheses that (1) ovarian number and development of the spermatheca covary in Cape workers, and (2) covary clinally as well. A regression analysis revealed that the frequencies of bees with spermathecae significantly decreased from west to east, with Stellenbosch having the highest and Grahamstown the lowest frequencies. Spermatheca size also significantly decreased from west to east. A multiple regression analysis revealed that the number of ovarioles significantly decreased from west to east and also differed significantly between workers with and without a spermatheca. Moreover, the number of ovarioles was significantly correlated with the size of the spermatheca and workers without it had significantly fewer ovarioles. Both hypotheses are confirmed. The outcomes of these measurements will provide a more quantitative basis for estimates of effective social parasitism among these bees along a geographic continuum.

***Apis mellifera capensis* / spermatheca / ovarioles**

1. INTRODUCTION

Workers of the Cape honeybee, *Apis mellifera capensis*, are facultative social parasites of other Cape colonies as well as the highland bee, *A. m. scutellata* (Neumann and Hepburn, 2002). This ability stems from their having the unique trait of thelytokous parthenogenesis in which workers lay diploid eggs (Anderson, 1963) and some have a well-developed spermatheca and a high number of ovarioles (Ruttner, 1976, 1977, 1988). However, the phenotypic expression of thelytoky is most certainly not an "all or none" characteristic and is actually geographically clinal (Hepburn and Crewe, 1991). "*A. m. capensis*" itself is a clinal spectrum of characters in which ovariole number, egg-laying pattern, the sex ratio of the progeny of laying workers, and the bouquet of the mandibular gland pheromone all gradually decrease over its 800 km natural range from east to west in coastal, southern South Africa (Hepburn et al., 1998). The completely clinal nature of numerous traits in this whole population provides a plausible

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* Manuscript editor: Yves Le Conte

theoretical background for enquiring if there is any correlation between frequency of ovarioles and spermatheca. The latter being normally a queen characteristic and the former playing a role in the reproductive success of an individual, so both are strongly related to reproductive dominance.

The mandibular gland pheromones and ovariole development covary in queenless Cape workers (Hepburn, 1992) (both probably under separate genetic control), but thelytoky itself is a Mendelian trait (Lattorff et al., 2005). Jordan et al. (2008) measured spermatheca size and number of ovarioles in the parental, hybrid and backcross offspring between thelytokous Cape bees and the highland bee *A. m. scutellata* and observed that they were phenotypically correlated but apparently under independent genetic control. Moreover, larval nutrition also affects spermatheca size and ovariole number (Beekman et al., 2000; Calis et al., 2002; Allsopp et al., 2003). Thus, both genetic and environmental effects influence spermathecal size, but less so the number of ovarioles (Jordan et al., 2008). The spermatheca issue in Cape workers is the potential link of these traits across the natural distribution range of Cape bees and the theoretical backbone of this investigation. In view of the extremely clinal nature of this honeybee population, we tested the hypotheses that (1) ovarian number and spermathecal development covary in Cape workers, and (2) covary clinally as well. The outcomes of these measurements will provide a more quantitative basis for estimates of effective social parasitism among these bees along a geographic continuum.

2. MATERIALS AND METHODS

2.1. Study sites and collection of specimens

A. m. capensis workers were collected from queenright colonies of about equal size at five sites at about 200 km intervals along an 800 km transect through the Western and Eastern Cape Provinces (South Africa) during February 2008: Stellenbosch (33.55S, 18.51E), Heidelberg (26.30S, 28.21E), George (33.58S, 22.26E), Jeffrey's Bay (34.20S, 24.55E) and Grahamstown (33.17S, 26.31E). A random sample of three hundred workers was collected from each of six colonies per site. A total of 9000 bees were collected, placed on ice and frozen for later dissection.

2.2. Dissections

All bees were dissected from the ventral side by carefully removing the middle and caudal abdominal sternites (Velthuis, 1970). Presence or absence of a spermatheca was recorded and, if present, it was measured. In addition, for each colony, ovaries from all workers with spermathecae and without spermathecae were removed and the total number of ovarioles was counted under a light microscope and photographed.

2.3. Statistical analysis

The minimum sample sizes required to estimate the true population proportions of workers with spermatheca at each location within a 5% error from the sample proportions with 95% confidence were calculated. Statistical significance was determined using analysis of variance (parametric) or Kruskal-Wallis (non-parametric) procedures to test for significant differences in the proportions of workers with spermatheca, size of spermatheca, and number of ovarioles in workers with and without spermathecae among the different localities at the 5% level of significance. Prior to analysis, homogeneity of variances and normality of the data were checked using Levene's tests and Shapiro-Wilks' tests (Johnson and Wichern, 2002). The proportions of workers with spermatheca among the localities failed tests of homogeneity of the variances and, therefore, nonparametric procedures were used. Means and standard errors were calculated. Simple and multiple regression analyses were used to determine the rate of decrease in the proportions of workers with spermatheca, and in the numbers of ovarioles in workers with and without spermathecae from Stellenbosch (west) to Grahamstown (east), respectively. All tests were performed using Statistica, version 8.0. (StatSoft, 2008).

3. RESULTS

Based on observed frequencies, the minimum sample sizes necessary to estimate the

Table I. Mean (±SE) percentages of Cape worker honeybees with spermatheca, and spermatheca size (N = 300 bees per colony, 6 colonies per locality).

Location	Percentage with spermatheca	Spermatheca size (mm)
Stellenbosch	19.2 ± 9.6	0.54 ± 0.01
Heidelberg	7.9 ± 4.8	0.46 ± 0.01
George	3.7 ± 1.7	0.48 ± 0.02
Jeffrey's Bay	5.3 ± 3.4	0.43 ± 0.01
Grahamstown	0.4 ± 0.2	0.41 ± 0.04

true proportions of workers with spermathecae within a 5% error from the sample proportions with 95% confidence were highly variable and for the different localities were: Stellenbosch $N_{min} = 238$, Heidelberg $N_{min} = 111$, George $N_{min} = 55$, Jefffereys Bay $N_{min} = 77$, and Grahamstown $N_{min} = 6$. The minimum sample sizes needed to estimate the true mean number of ovarioles in workers with and without spermathecae within a 5% error from the sample mean ovariole number ranged from 134 to 297.

3.1. Presence/absence of the spermatheca

The worker spermatheca, when present and developed, is fully invested with a tracheolar net and spermathecal gland and is connected to the oviduct (Fig. 1) as previously illustrated by both Fyg (1950) and Ruttner (1988).

There was a significant difference in the percentages of spermathecae among the localities (Kruskal-Wallis ANOVA: H (4, $N = 30$) = 9.7, $P = 0.0460$, Tab. I). A regression analysis revealed that the frequencies of bees with spermathecae significantly decreased from west to east, with Stellenbosch having the highest and Grahamstown the lowest frequencies ($F_{1,28} = 9.2$, $P < 0.0051$). The Mann-Whitney post-hoc comparisons confirmed that the percentages of spermathecae between these two localities were significantly different ($U = 2.5$, $P < 0.0113$). Spermatheca size also significantly decreased from west to east ($F_{4,652} = 21.8$, $P < 0.0001$, Tab. I).

3.2. Number of ovarioles

There were significant differences in the mean number of ovarioles among the localities (ANOVA: $F_{4,1456} = 19.0$, $P < 0.0001$) and between workers with and without a spermatheca (ANOVA: $F_{1,1456} = 4.2$, $P < 0.0399$, Fig. 2). There was no significant interaction effect between the localities and with/without spermathecae (ANOVA: $F_{4,1456} = 1.4$, $P = 0.2152$). A multiple regression analysis revealed that the number of ovarioles significantly decreased from west to east and also differed significantly between workers with and without a spermatheca (Multiple regression analysis: $F_{2,1473} = 83.2$, $P < 0.0001$; west to east, $ß_1 = -0.176$, $t_{1473} = -9.8$, $P < 0.0001$, spermatheca present or not, $ß_2 = 0.206$, $t_{1473} = 3.9$, $P < 0.0001$). Moreover, the number of ovarioles was significantly correlated with the size of the spermatheca (correlation: $r = 0.22$, $P < 0.01$, $N = 606$).

4. DISCUSSION

Jordan et al. (2008) investigated the genetic background for spermatheca occurrence and number of ovarioles in the parental, hybrid and backcross offspring between thelytokous Cape bees and the highland bee *A. m. scutellata* and observed that these traits were phenotypically correlated but apparently under independent genetic control. This was an important result, but there was no data on the natural frequency of occurrence of these two traits within the Cape bee population which was the motivation for the current study. The results of the survey clearly showed significant clinal decreases in the frequency and size in spermatheca in *A. m. capensis* workers along the west-to-east transect.

Ovariole numbers in workers with spermathecae were significantly higher than in workers without spermathecae and this is interpreted as probably expressing more "queen genes" than those without a well developed spermatheca because one would expect a higher number of ovarioles in the former. That the frequency and size of spermatheca is negatively correlated with the distance from Cape

Figure 1. The developed worker spermatheca of *A. m. capensis* is invested with a tracheolar net and the spermathecal gland is connected to the oviduct.

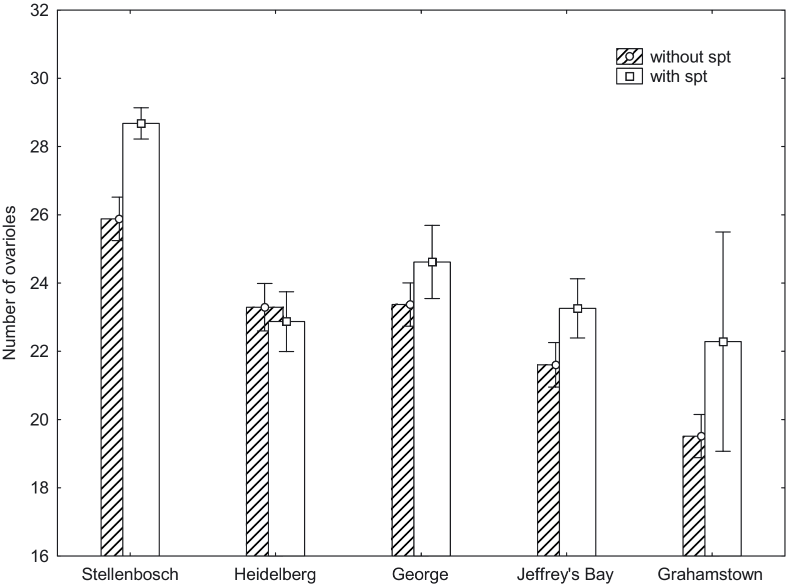

Figure 2. Mean (±SE) number of ovarioles in Cape worker honeybees with and without spermathecae at each locality.

Town is completely consistent with existing knowledge that traits *of A. m. scutellata* are gradually replaced by those of *A. m. capensis* (Hepburn, 1992; Hepburn et al., 1998).

Likewise, ovariole numbers in workers with spermathecae and those without spermathecae also decreased in the same direction along the same cline. Moreover, ovariole number is significantly positively correlated with spermatheca size. These results confirm and extend the clinal geographical distribution suggested by the data of Hepburn and Crewe (1991).

It is of interest to note that both ovariole number and spermatheca size are both affected by larval nutrition (Beekman et al., 2000; Calis et al., 2002; Allsopp et al., 2003). However, these elements do not mask the genetic basis or phenotypic expression of these traits because Jordan et al. (2008) showed unequivocally that spermatheca size and number of ovarioles in the parental, hybrid and backcross offspring were both phenotypically correlated but apparently under independent genetic control. However, given the narrow window of sampling we used, we believe that any environmental effects would have been minimal.

By the same token Hepburn (1992) showed that the transition of mandibular gland pheromones in queenless capensis workers and ovariole development covary and suggested that both were probably under separate genetic control. Given that thelytokous parthenogenesis in Cape workers was shown to be a Mendelian trait (Lattorff et al., 2005) and that spermatheca size and number of ovarioles can both be interpreted parsimoniously as under single allele control, it begins to appear that the complex reproductive capacity of Cape workers may well be little more than a half dozen independent genes somehow working together. Thus, there is a series of correlations for the amplification of worker reproductive organs into a more queen-like state and these are distributed in an entirely clinal manner, decreasing from west-to-east. The clinal changes observed reasonably imply a similar clinal decrease in the frequencies of these genes over time and space.

The observed relationships between several traits related to reproductive dominance suggest that the Cape honeybee constitutes a throwback, displaying an ancestral phenotype, when presumably honeybee queens and workers were similar than nowadays. That atavism of the presents of a spermatheca might be caused by the evolutionary pressure for reproductive dominance in *A. m. capensis*, making that subspecies an ideal model for investigating the underlaying mechanism of the evolution of sociality and caste differentiation.

ACKNOWLEDGEMENTS

Kendall Crous, Yang Mingxian and Huo-Quing Zheng are thanked for assistance with field work and dissections of bees and Theresa Wossler for some honeybee samples. We thank Peter Neumann for his constructive criticisms of an earlier draught of this manuscript.

Nature clinale de la fréquence des ovarioles et des spermathèques observée chez les ouvrières de l'abeille du Cap, *Apis mellifera capensis*.

***Apis mellifera capensis* / variation géographique / Afrique du Sud / spermathèques / ovarioles**

Zusammenfassung – **Klinale Veränderung der Häufigkeiten von Ovariolen und Spermatheken bei Arbeiterinnen der Kaphonigbiene, *Apis mellifera capensis*.** Um bei der Kaphonigbiene, *Apis mellifera capensis*, eine Einschätzung des Anteils der Arbeiterinnen mit Spermatheken mit einer statistischen Sicherheit von 95 % zu erreichen wurde festgestellt, dass ein Probenumfang von 300 Arbeiterinnen benötigt wird. Proben dieser Größe wurden in Südafrika entlang eines 800 km langen Transects durch die westliche und östliche Kapprovinz an 5 je 200 km voneinander entfernten Orten von jeweils 6 Bienenvölkern pro Sammelort gesammelt. Ausgehend von der extrem klinalen Natur dieser Bienenpopulation prüften wir die Hypothesen dass (1) die Zahl der Ovarien mit der Entwicklung der Spermatheka kovariiert, und dass (2) diese Kovarianz klinal ausgeprägt ist. Die Bienen wurden präpariert, um die Häufigkeit und Größe der Spermatheken sowie die Zahl der Ovariolen bei Arbeiterinnen mit bzw. ohne Spermatheka festzustellen. Ausgehend von den beobachteten Häufigkeiten war der mindestens benötigte Probenumfang zur Bestimmung des Anteils von Arbeiterinnen mit Spermatheka mit einer Irrtumswahrscheinlichkeit von 5 % sehr variabel und für die verschiedenen Orte wie folgt: Stellenbosch $N_{min} = 238$, Heidelberg $N_{min} = 111$, George $N_{min} = 55$, Jeffereys Bay $N_{min} = 77$, und Grahamstown $N_{min} = 6$. Die mindestens benötigte

Probengröße zur Bestimmung der tatsächlichen Anzahl der Ovariolen bei Arbeiterinnen mit bzw. ohne Spermatheka mit einer Irrtumswahrscheinlichkeit von 5 % lag zwischen 134 und 297.

Die natürliche geographische Verteilung beeinflusst die Häufigkeit und Größe der Spermatheken und die Anzahl der Ovariolen bei Arbeiterinnen der Kapbiene. Die Unterschiede in der Häufigkeiten von Spermatheken zwischen den verschiedenen Orten waren signifikant (Kruskal-Wallis ANOVA: H(4, N = 30) = 9,7, P = 0,0460, Tab. I). Mittels einer Regressionsanalyse wurde festgestellt, dass die Häufigkeit von Bienen mit Spermatheka von West nach Ost signifikant abnahm, wobei in Stellenbosch die größte und in Grahamstown die niedrigste Häufigkeit gefunden wurde ($F_{1,28}$ = 9,2, $P < 0{,}0051$). Der Mann-Whitney post-hoc Vergleich bestätigte, dass die prozentuale Häufigkeit von Spermatheken zwischen diesen beiden Orten signifikant verschieden war (U = 2,5, $P < 0{,}0113$). Ebenso nahm die Größe der Spermatheken von West nach Ost signifikant ab ($F_{4,652}$ = 21,8, $P < 0{,}0001$, Tab. I).

Die Unterschiede in der mittleren Anzahl von Ovariolen zwischen den Orten (ANOVA: $F_{4,1456}$ = 19,0, $P < 0{,}0001$) und zwischen Arbeiterinnen mit und ohne Spermatheka (ANOVA: $F_{1,1456}$ = 4,2, $P < 0{,}0399$, Abb. 2) waren signifikant. Es wurde kein signifikanter Interaktionseffekt zwischen den Orten und dem Vorhandensein bzw. Fehlen von Spermatheken gefunden (ANOVA: $F_{4,1456} = 1,4$, $P = 0,2152$). Eine multiple Regressionsanalyse zeigte, dass die Anzahl der Ovariolen von West nach Ost signifikant abnahm und sich bei Arbeiterinnen mit und ohne Spermatheka signifikant unterschied (Multiple Regressionsanalyse: $F_{2,1473}$ = 83,2, $P < 0{,}0001$; West nach Ost, $ß_1$ = –0,176, t_{1473} = –9,8, $P < 0{,}0001$, Spermatheka vorhanden bzw. fehlt, $ß_2$ = 0,206, t_{1473} = 3,9, $P < 0{,}0001$). Darüber hinaus war die Anzahl der Ovariolen signifikant mit der Größe der Spermatheka korreliert (Korrelation: r = 0,22, $P < 0{,}01$, N = 606). Somit gibt es eine Reihe von Korrelationen für die Verstärkung von reproduktiven Organen bei Arbeiterinnen, hin zu einem Status, der mehr einer Königin entspricht. Dies geschieht in einer vollständig klinalen Weise und nimmt von West nach Ost ab. Diese beobachteten klinalen Änderungen lassen daher den Schluss auf eine ähnliche klinale Abnahme der Häufigkeiten dieser Gene in lokalen Populationen über Raum und Zeit zu.

***Apis mellifera capensis* / Spermatheken / Ovariolen**

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