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Original article

A method for estimating variation in the phenotypic expression of morphological characters by thelytokous parthenogenesis in *Apis mellifera capensis*

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Abstract – Thelytokous parthenogenesis in Cape worker honeybees, *Apis mellifera capensis*, was used to produce a series of clonal progeny that were reared in three different, queenless arrhenotokous *A. m. scutellata* host colonies. Each individual Cape worker bee was genotyped at 4 DNA microsatellite loci to verify its clonal status and measured for 36 morphological characters. The clonal workers bees, all of the same thelytokous matriline, were then analysed by multivariate analysis to determine the quantitative effects of environment on the morphological characters. This in turn allows the estimation of the natural variation in the phenotypic expression of morphological characters. Coefficients of environmental variation were calculated and the relative stability of the character set was, in decreasing order, body size, forewings, wing venation, hairs and pigmentation.

***Apis mellifera capensis* / clone / honeybee / morphometrics / environmental variance / thelytoky**

1. INTRODUCTION

Multivariate analyses of morphometric characters are used to delimit variation in natural populations of honeybees (*Apis mellifera* L.). The accuracy and predictive value of these procedures depend on measurement quality as well as the quantitative relationship between the genotype and phenotype as modified by environmental effects (Falconer, 1989). The susceptibility of morphological characters to effects of

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environment was recognised long ago (Alpatov, 1929) and remains under scrutiny (Daly et al., 1991; Nazzi, 1992). Environmentally induced variation vis-a-vis genetic control of character expression are not easily separated (Thorpe, 1976).

Moreover, the heritability of morphological characters is rather variable (Rinderer, 1977; Rinderer et al., 1990; Poklukar and Kezic, 1994), as confirmed by both sib analysis as well as parent offspring regression methods (Moritz and Klepsch, 1985; Oldroyd et al., 1991). Of particular interest in this context, Oldroyd et al. (1991) demonstrated that apparent values of heritability actually declined when bees were raised under cross-fostered conditions.

The parent-offspring method of analysis, based on thelytokous parthenogenesis (production of diploid females by unmated laying workers), is a particularly useful probe because geographical or environmental variation in morphometric characters can be deduced from the fact that the offspring are all clonal, isogenic offspring of a single laying worker. Thus, intercolonial variation among cloned honeybees must reflect whatever environmental influences are operative whether they can be specified or not (Sokal et al., 1980; Moritz and Klepsch, 1985). By experiment and calculation it is thus possible to derive both coefficients of environmental variation as well as genetic residuals of morphological variation for natural populations (Hepburn et al., unpublished data).

Although occasional recombination events may occur in thelytoky through crossing-over and segregation of genes (Slobodchikoff and Daly, 1971), they are highly improbable in thelytokously reproducing honeybees (Moritz and Haberl, 1994). Thus, rather precise control over the genotype can be achieved and measured for clonal offspring produced by such laying workers, making it possible to quantify both environmental and genetic components of morphometric variation (Hepburn and Radloff, 2002; Hepburn et al., unpublished data).

2. MATERIALS AND METHODS

2.1. Sampling

Three queenless colonies of *Apis mellifera scutellata* Lepeletier infested with *Apis mellifera capensis* Escholtz laying workers (for biological details see Calis et al., 2002; Martin et al., 2002; Moritz, 2002; Neumann and Hepburn, 2002; Pirk et al., 2002; Reece, 2002; Wossler, 2002) were obtained from Pretoria, South Africa. Once all of the sealed worker brood of the former *A. m. scutellata* host queens emerged as adults, the colonies were monitored for laying worker brood of *A. m. capensis* (Calis et al., 2002). After this thelytokously produced worker brood was sealed, brood frames were removed from the colonies, individually confined in gauze-covered cages and placed in an incubator until adult emergence. After a week of feeding ad libitum (so that expansion and hardening of the exoskeleton was complete) the newly emerged adults of the laying worker brood were individually coded and genetically analysed to establish whether they originated from the same laying worker matriline.

2.2. DNA analysis

DNA was extracted from 20 workers of each colony and genotyped at four DNA microsatellite loci A107, A24, A28, and A43 (Estoup et al., 1993, 1994, 1995) according to routine protocols Neumann et al. (1999a,b,c).

2.3. Morphological measurements

Thirty-five morphological characters (standard in honeybee morphometry (Ruttner, 1988; Hepburn and Radloff, 1998),

Table I. Means and intra- and intercolonial standard deviations (sd) of the 36 morphometric characters of workers from colonies C1-3.

Character	C1		C2		C3			Significance level	
	mean	intra sd	mean	intra sd	mean	intra sd	inter sd	mean	variance
(1)	0.135[a]	0.016	0.123[a]	0.018	0.123[a]	0.018	0.007	ns	ns
(2)	0.392[a]	0.086	0.341[b]	0.098	0.417[a]	0.081	0.039	*	ns
(3)	0.711[a]	0.111	0.687[a]	0.148	0.749[a]	0.092	0.031	ns	ns
(5)	2.515[a]	0.049	2.556[b]	0.044	2.556[b]	0.039	0.024	*	ns
(6)	3.078[a]	0.064	3.096[a]	0.045	3.077[a]	0.063	0.011	ns	ns
(7)	1.914[a]	0.044	1.940[a]	0.037	1.915[a]	0.033	0.015	ns	ns
(8)	1.132[a]	0.031	1.132[a]	0.039	1.123[a]	0.035	0.005	ns	ns
(9)	2.103[a]	0.047	2.108[a]	0.063	2.082[a]	0.066	0.014	ns	ns
(10)	2.046[a]	0.036	2.053[a]	0.069	1.998[b]	0.063	0.030	*	ns
(11)	2.659[a]	0.039	2.646[ab]	0.073	2.594[b]	0.087	0.034	*	ns
(12)	1.203[ab]	0.034	1.234[a]	0.044	1.189[b]	0.052	0.023	*	ns
(13)	2.118[ab]	0.048	2.159[a]	0.061	2.116[b]	0.050	0.024	*	ns
(14)	0.304[a]	0.061	0.311[a]	0.047	0.293[a]	0.056	0.009	ns	ns
(15)	2.681[a]	0.049	2.676[a]	0.045	2.652[a]	0.038	0.016	ns	ns
(16)	2.848[a]	0.059	2.847[a]	0.037	2.797[b]	0.044	0.029	*	ns
(17)	8.831[a]	0.181	8.928[a]	0.110	8.864[a]	0.121	0.049	ns	ns
(18)	2.979[a]	0.118	3.016[a]	0.106	2.986[a]	0.081	0.020	ns	ns
(19)	0.547[a]	0.021	0.552[a]	0.021	0.546[a]	0.024	0.003	ns	ns
(20)	0.232[a]	0.016	0.231[a]	0.015	0.237[a]	0.016	0.003	ns	ns
(21)	29.900[a]	1.680	28.776[a]	1.181	28.768[a]	1.571	0.651	ns	ns
(22)	104.95[a]	5.068	108.00[a]	2.346	107.86[a]	3.150	1.722	ns	ns
(23)	103.68[a]	2.323	104.64[a]	2.429	103.42[a]	2.191	0.643	ns	ns
(24)	19.912[a]	1.073	20.459[a]	0.884	20.010[a]	0.983	0.292	ns	ns
(25)	104.10[a]	2.323	103.07[a]	3.219	102.39[a]	2.659	0.861	ns	ns
(26)	74.262[a]	3.343	73.906[ab]	3.640	71.637[b]	2.505	1.424	*	ns
(27)	20.206[a]	1.531	20.276[a]	1.402	19.963[a]	1.649	0.164	ns	ns
MJI	90.475[a]	2.823	87.235[b]	2.356	88.158[b]	2.842	1.669	*	ns
(28)	80.212[a]	1.433	79.559[a]	1.255	80.600[a]	3.690	0.526	ns	ns
(29)	12.181[a]	0.830	12.070[a]	0.822	12.663[a]	1.398	0.315	ns	*
(30)	86.600[a]	2.031	86.012[a]	3.111	85.679[a]	2.514	0.466	ns	ns
(31)	33.356[a]	3.556	31.082[a]	2.177	31.174[a]	2.986	1.287	ns	ns
(32)	5.000[a]	0.000	4.823[a]	0.528	5.000[a]	0.000	0.102	ns	ns
(33)	5.000[a]	0.000	4.823[a]	0.528	4.947[a]	0.229	0.091	ns	ns
(34)	3.375[a]	0.619	3.529[a]	0.514	3.474[a]	0.513	0.078	ns	ns
(35)	0.687[a]	0.873	1.294[a]	0.919	0.789[a]	0.976	0.325	ns	ns
(36)	1.187[a]	0.544	1.412[a]	0.507	1.105[a]	0.567	0.159	ns	ns

* $P < 0.05$; ** $P < 0.01$; ns = not significant; a,b = different letters within a row indicate significant mean differences ($P < 0.05$).

were measured from workers of three colonies (n = 16, 17, and 19 respectively per colony) which were offspring of the same matriline (Tab. I). Their Ruttner (1988) numbers are given in brackets as follows: length of cover hair on tergite 5 (1), width of tomentum on tergite 4 (2), width of stripe posterior of tomentum (3), length of femur (5), length of tibia (6), metatarus length (7), metatarsus width (8), tergite 3 longitudinal (9), tergite 4, longitudinal (10), sternite 3, longitudinal (11), wax plate of sternite 3, longitudinal (12) wax plate of sternite 3, transversal (13), distance between wax plates, sternite 3 (14), sternite 6, longitudinal (15), sternite 6, transversal (16), forewing, longitudinal (17), forewing, transversal (18), cubital vein, distance a (19), cubital vein, distance b (20), wing angle A4 (21), wing angle B4 (22), wing angle D7 (23), wing angle E9 (24), wing angle G18 (25), wing angle I10 (26), wing angle I16 (27), wing angle K19 (28), wing angle L13 (29), wing angle N23 (30), wing angle O26 (31), pigmentation of tergite 2 (32), pigmentation of tergite 3 (33), pigmentation of tergite 4 (34), pigmentation of scutellum (35), pigmentation of scutellar plate (36). In addition, the wing angle MJI (cf. Ruttner, 1988; Fig. 6.9) was measured.

2.4. Data analysis

Univariate analyses were carried out on the means, standard deviations and coefficients of variation for all morphological characters. Differences between colonies for the means and variances were tested using ANOVA with Scheffé multiple comparison tests and Levene's procedures (Rao, 1998). The Kolmogorov-Smirnov test was used to test for normality of the distribution of the coefficients of variation. Non-parametric Kruskal-Wallis, Wilcoxon matched pairs and Mann-Whitney tests were used to test for significant differences of the coefficients of variation between colonies and morphometric characters. Subsequent multivariate analyses included factor and discriminant analyses to determine morphometric differences between the colonies (Johnson and Wichern, 1998).

It must be noted that the coefficient of variation may not be stable nor representative for different kinds of measurements. While appropriate for length measures, in the case of pigmentation had the convention been to give high scores for "black" instead of "yellow" then any ranking of coefficients of variation might seem arbitrary. However, the reason for the measure of the coefficient of variation is to make measures of variation independent of the size (values) of the characters being measured, hence allowing comparisons. The drawbacks of the coefficients of variation do not directly nor indirectly bear on the calculation of the natural variances. If, for example, the bees were given codes so that for y values: "black" = 9 and "yellow" = 0 and then for x values: "black" = 0 and "yellow" = 9 (these are Ruttner, 1988 codes) the variances for both would be exactly the same; that is, y = 9-x hence var(y) = var(x). Of course the means would be different so that mean(y) = 9 - mean(x) and hence the coefficients of variation would be different.

3. RESULTS

The genotypes of all workers (allele sizes in base pairs), which were included in the morphometric analysis, were determined by the analysis of four DNA microsatellite loci A107 (176 bp, 181 bp), A24 (90 bp, 94 bp), A28 (131 bp, 131 bp) and A43 (121 bp, 142 bp) and thus shown to derive from the same matriline.

Hypothesis tests for differences between colony means for each character showed that 25% (9/36) of them were significantly different ($P < 0.05$) and 75% (27/36) were not (Tab. I). Levene's tests for differences between colony variances for each character individually revealed that 3% (1/36) of

Table II. Coefficients of environmental variation of the 36 morphometric characters of workers from colonies C1-3.

Character	Colony			
	C1	C2	C3	Pooled
F(17)	0.0205	0.0123	0.0136	0.0157
S(15)	0.0184	0.0168	0.0142	0.0165
S(16)	0.0207	0.0129	0.0156	0.0166
S(5)	0.0196	0.0173	0.0155	0.0174
S(6)	0.0208	0.0144	0.0205	0.0188
S(7)	0.0228	0.0190	0.0172	0.0193
A(23)	0.0224	0.0232	0.0212	0.0222
S(13)	0.0225	0.0284	0.0236	0.0249
A(25)	0.0223	0.0312	0.0259	0.0268
S(11)	0.0146	0.0278	0.0335	0.0269
S(9)	0.0225	0.0298	0.0319	0.0286
S(10)	0.0174	0.0337	0.0317	0.0288
A(30)	0.0234	0.0362	0.0293	0.0302
MJI	0.0312	0.0270	0.0322	0.0303
A(28)	0.0179	0.0158	0.0458	0.0309
S(8)	0.0278	0.0343	0.0311	0.0312
F(18)	0.0397	0.0352	0.0272	0.0341
A(22)	0.0483	0.0217	0.0292	0.0341
S(12)	0.0287	0.0358	0.0442	0.0372
F(19)	0.0386	0.0382	0.0441	0.0406
A(26)	0.0450	0.0493	0.0349	0.0433
A(24)	0.0539	0.0432	0.0491	0.0488
A(21)	0.0562	0.0410	0.0546	0.0512
P(32)	0.0000	0.1096	0.0000	0.0611
F(20)	0.0690	0.0633	0.0690	0.0673
P(33)	0.0000	0.1096	0.0464	0.0675
A(27)	0.0758	0.0692	0.0826	0.0762
A(29)	0.0681	0.0681	0.1104	0.0870
A(31)	0.1066	0.0700	0.0958	0.0927
H(1)	0.1163	0.1455	0.1488	0.1374
P(34)	0.1834	0.1458	0.1477	0.1583
H(3)	0.1569	0.2155	0.1227	0.1654
S(14)	0.2025	0.1509	0.1913	0.1821
H(2)	0.2200	0.2878	0.1949	0.2305
P(36)	0.4580	0.3593	0.5131	0.4396
P(35)	1.2701	0.7106	1.2367	1.0045

A = angles of wing venation, S = size, H = hair, P = pigmentation, F = forewing.

the characters showed heterogeneous variation whilst 97% (35/36) were uniform and showed no significant differences (Tab. I).

The distribution of the coefficients of variation failed the test of normality and hence non-parametric procedures were used in the analysis ($d = 0.37$, $P < 0.01$). No significant differences were found between colonies for the coefficients of variation (Tab. II; $\chi^2 = 1.67$, 2df, $P = 0.8678$; Wilcoxon matched pairs tests: C1 and C2, $T = 305.0$, $P = 0.8698$; C1 and C3, $T = 296.0$, $P = 0.9795$; C2 and C3, $T = 258.00$, $P = 0.2387$). Factor and discriminant analyses revealed a single morphocluster for the three colonies.

However, the susceptibility to environmental modification of different categories of morphological characters is variable, so that rank order may not necessarily be stable if environmental effects are variable. To consider this aspect, the 36 characters examined were classified in five categories of varying stability: A = angles of wing venation, S = size, H = hair, P = pigmentation and F = forewing and further analysed by groups. The means and standard deviations of these groups were: $A = 0.0478 \pm 0.0245$, $S = 0.0374 \pm 0.0461$, $H = 0.1778 \pm 0.0478$, $P = 0.3462 \pm 0.3989$ and $F = 0.0394 \pm 0.0214$ respectively. Kruskal-Wallis test procedure shows that there is an overall highly significant difference among the five categories ($H = 18.06$, (4, $n = 36$), $P = 0.0012$) and that the pigmentation group (P) is indeed significantly more variable than the other character groups (Mann-Whitney $U < 6.0$, $P < 0.05$).

4. DISCUSSION

The experimental data clearly demonstrate that thelytokous parthenogenesis can unequivocally differentiate the various components of phenotypic variation. The data also show that the effect of environment on the different morphometric characters is significantly different among them. Thus, without knowing the precise environmental effect on each individual character, this would represent a non-conservative systematic error, resulting in a conflation of the population data. Coupled to multivariate analyses of morphometric characters, fine resolution of genetic components becomes possible.

It is of interest to consider the separate contributions of intra- and intercolonial variation in the phenotypic expression of morphometric characters. Table I clearly illustrates that intracolonial variation is considerably greater than intercolonial variation for all morphometric characters. Owing to the mathematical procedures to derive intercolonial values this could be expected to remain so were the number of colonies increased beyond the three discussed here.

The results further establish through the magnitudes of the coefficients of variation which of the 36 morphological characters are most conserved (least sensitive to environmental effects). This is highly important both to the analysis of natural populations as well as for honeybee intraspecific classification. Clearly the construction of population profiles and classification paradigms are mutually interdependent, the quality of any inferences being dependent on the characters considered.

In this respect the metrical characters for size are most useful in population analyses, the more subjective ones relating to pigmentation considerably less so. Of the 36 characters measured in this study 23 of them, all metrical, probably have generally high heritability values (Moritz and Klepsch, 1985; Cornuet and Garnery, 1991; Oldroyd et al., 1991). Morphological traits with low heritability (e.g. number of legs in adult worker bees) are constant and are therefore of no use for population studies. However, traits with high heritability will almost certainly be variable in a population. So for 23 traits the impact of environmental

effects on the isogenic groups is small. Given the successfulness of thelytokous parthenogenesis as a genetic probe, in future it is possible to design experiments that simultaneously and quantitatively control all aspects of the genetic equation required to move from genotype through environmentally modulated phenotype.

Résumé – Méthode pour estimer la variation de l'expression phénotypique des caractères morphologiques dans la parthénogenèse thélytoque chez *Apis mellifera capensis.* Les ouvrières pondeuses de l'abeille du Cap (*Apis mellifera capensis* Escholtz) produisent une descendance femelle par parthénogenèse thélytoque, ce qui permet de quantifier les effets de l'environnement sur l'expression du génotype dans les lignées clonales. La descendance d'une lignée clonale d'ouvrières pondeuses thélytoques a été élevée dans trois colonies hôtes d'*Apis mellifera scutellata* arrhénotoques (les ouvrières pondeuses produisent des mâles) et orphelines (différents environnements). Le génotype de chaque abeille du Cap ainsi obtenue a été caractérisé à 4 locus de microsatellites d'ADN et 36 caractères morphologiques ont été mesurés. Les abeilles clonales de la même lignée maternelle thélytoque ont été ensuite analysées pour déterminer les effets quantitatifs de l'environnement sur les caractères morphologiques par l'analyse multivariée. Les génotypes de toutes les ouvrières ont confirmé que toutes les abeilles testées des trois colonies provenaient de la même lignée maternelle. On a réalisé des analyses ANOVA pour déterminer avec précision l'étendue de la variance du milieu pour chacun des caractères morphologiques. Les moyennes étaient significativement différentes pour 25 % des caractères et les variances pour 3 % (Tab. I) et il n'y avait pas de différences significatives entre colonies pour les coefficients de variation (Tab. II). L'analyse factorielle et discriminante a fourni un seul morphogroupe pour ces abeilles. Les coefficients de variation ont fourni un ordre de classement de la sensibilité des caractères aux effets du milieu (Tab. II) par rapport à un fond génétique maintenu constant. Les groupes de caractères les plus utiles sont par ordre décroissant : la taille corporelle, les ailes antérieures, la vénation alaire, la pilosité et la pigmentation. Ainsi la parthénogenèse thélytoque chez *A. m. capensis* couplée aux analyses morphométriques multivariées permet de mesurer les effets du milieu sur l'expression phénotypique des caractères et de les séparer des effets génotypiques.

***Apis mellifera capensis* / clone / morphométrie / parthénogenèse thélytoque / variabilité du milieu**

Zusammenfassung – Abschätzung natürlicher Variation in der phänotypischen Ausprägung morphometrischer Merkmale durch thelytoke Parthenogenese in *Apis mellifera capensis.* Legende Arbeiterinnen der Kaphonigbiene können klonalen weiblichen Nachwuchs produzieren (Thelytokie). Von daher ist es möglich, umweltbedingte Effekte auf genotypische Expression in klonalen Linien zu quantifizieren. Nachkommen einer klonalen Linie von thelytoken legenden Arbeiterinnen wurden in drei verschiedenen weisellosen arrhenotoken (legende Arbeiterinnen produzieren Drohnen) *A. m. scutellata* Wirtsvölkern aufgezogen (verschiedene Umweltbedigungen). Jede individuelle Kaparbeiterin wurde an vier DNA microsatellite Loci genotypisiert und morphometrisch für 36 Merkmale untersucht. Die klonalen Nachkommen derselben thelytoken Matrilinie wurden dann mit Hilfe multivariater Statistik analysiert, um die quantitativen umweltbedingten Effekte auf die morphometrischen Merkmale zu bestimmen.
Die Genotypen der untersuchten Arbeiterinnen zeigten, dass alle untersuchten Bienen in den drei untersuchten Völkern von einer einzigen Matrilinie stammten. ANOVA wurden für jedes untersuchte morphometrische

Merkmal durchgeführt, um das Ausmaß an unweltbedingter Varianz abzuschätzen. 25 % der untersuchten Merkmale wiesen signifikant verschiedene Mittelwerte und 3 % signifikant verschiedene Varianzen auf (Tab. I). Es gab keine signifikant unterschiedlichen Variationskoeffizienten zwischen den Völkern (Tab. II). Faktor und Diskriminanz Analysen zeigten einen einzigen Morphocluster für die untersuchten Bienen auf. Die Variationskoeffizienten zeigten eine Rangordnung in Bezug auf die Sensibilität gegenüber umweltbedingten Effekten (Tab. II). Die am wenigsten sensitiven Merkmalsgruppen waren in abnehmender Reihenfolge: (1) Merkmale der Körpergröße (2) der Vorderflügel (3) Flügeläderung (4) Haare (5) Pigmentierung. Die Kombination aus thelytoker Parthenogenese und multivariater Analyse morphometrischer Merkmale bei der Kaphonigbiene erlaubt es, umweltbedingte Effekte auf die phänotypische Merkmalsausprägung abzuschätzen und von genotypischen Effekten zu separieren.

***Apis mellifera capensis* / Klone / Honigbienen / Morphometrie / Umweltvariabilität / Thelytokie**

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