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Original article

Environmentally-induced developmental effects on morphometric characters of workers in *Apis cerana* colonies*

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Abstract – Experiments were performed to tease out the possible effects of single and mixed-species colonies (*Apis cerana* and *A. mellifera ligustica*) on morphological development in *A. cerana* workers. The graph of principal components scores revealed three distinct morphological clusters: (A) *A. cerana* in *A. cerana* cells reared by *A. cerana* worker bees (*cerana* control group); (B) *A. cerana* in *A. mellifera* cells reared by *A. cerana* (*cerana* cell-test group); (C) *A. cerana* in *A. mellifera* cells reared by mixed *A. cerana* and *A. mellifera* (*cerana* test group), *A. mellifera* in *A. mellifera* cells reared by *A. mellifera* (*mellifera* control group) and *A. mellifera* in *A. cerana* cells reared by *A. mellifera* (*mellifera* cell-test group). It is shown that *A. cerana* worker bees larger in size than normal can be produced both by rearing them in larger cells and also by rearing them in mixed-species colonies.

***Apis cerana* / *Apis mellifera* / morphometrics / development / mixed-species colonies**

1. INTRODUCTION

There are numerous developmental constraints, which can affect phenotypic expression in honeybees. Amongst the most intuitively obvious of these are cell size (Grout, 1937) and nutrition (Haydak, 1943, 1970). Nonetheless, the relative effects of these two possible environmental factors have not yet been teased apart experimentally. The opportunity to shed further light on this problem arose following the successful development of mixed-species colonies of *A. cerana* and *A. mellifera* (Tan et al., 2006). Mated queens and workers of *A. cerana* and *A. mellifera* can be reciprocally exchanged, but introductions of larvae have usually failed (Oschmann, 1965; Dhaliwal and Atwal, 1970; Adlakha and Sharma, 1971; Oku and Ono, 1990; Potichot et al., 1993). Although *A. mellifera* colonies would not rear *A. cerana* larvae, the latter could be reared into adult workers in vitro with *A. mellifera* royal jelly (Oku and Ono, 1990). The experiments of Tan et al. (2006) unequivocally established that morphological "mega-queens" were produced in mixed-species colonies and they attributed the effect in part to *A. mellifera* nurse bees feeding *A. cerana* larvae. Coupling the results of Oku and Ono (1990) and Tan et al. (2006), we designed an experiment to determine the

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Table I. Experimental test regime.

Treatment No.	Treatment name	Focal bees	Cell type	Reared by
1	*cerana* control	*cerana*	*cerana*	*cerana*
2	*cerana* test (mixed rearing + large-cell)	*cerana*	*mellifera*	*cerana* + *mellifera*
3	cell-test (larger)	*cerana*	*mellifera*	*cerana*
4	*mellifera* control	*mellifera*	*mellifera*	*mellifera*
5	cell-test (smaller)	*mellifera*	*cerana*	*mellifera*

effects of single and mixed-species colonies (*A. cerana* and *A. mellifera ligustica*) and differences in worker cell sizes on morphological development of *A. cerana* workers.

2. MATERIALS AND METHODS

2.1. Experiments

Worker bees were collected from five experimental groups (cf. Tab. I): (1) two normal colonies (1, 2) of *A. cerana* in which the brood was reared by *A. cerana* workers in *A. cerana* cells constitute the *cerana* control group (15 bees per colony, N = 30); (2) two combs of sealed *A. mellifera* brood in *A. mellifera* cells (~1500 cells) were then introduced into each of these same two colonies (now 1 has become 3 and 2 become 4) and when the *A. mellifera* brood eclosed as adults, the *A. cerana* queen laid in the empty *A. mellifera* cells and this generation of *A. cerana* brood was reared both by the newly eclosed *A. mellifera* as well as by *A. cerana* workers (*cerana* test group, 15 bees per colony, N = 30); (3) two normal colonies (5, 6) of *A. cerana* which were given *A. mellifera* combs in which the queens laid and this brood was reared by normal *A. cerana* workers (*cerana* cell-test group, 15 bees per colony, N = 30); (4) two normal colonies (7, 8) of *A. mellifera* in which normal *A. mellifera* brood was reared by normal *A. mellifera* workers in normal *A. mellifera* cells (*mellifera* control group, 15 bees per colony, N = 30); (5) two normal *A. mellifera* colonies (now 7 has become 9 and 8 become 10) which were given *A. cerana* combs in which the queens laid and this brood was reared by normal *A. mellifera* workers (*mellifera* cell-test group, 15 bees per colony, N = 30). In treatment 2 the ratio of the mixed-species adult bees was about 1:5 *mellifera*/*cerana*. Workers were collected into ethanol and preserved for measurement.

2.2. Measurements

To evaluate the possible effects of mixed-species colonies and two cell sizes of worker comb, measurements were performed using a stereo-microscope and a computer-aided measuring system based on a video system and measuring program (Meixner, 1994). The morphological characters of worker bees were measured using the methods of Ruttner (1988) and the Ruttner parameters and their Ruttner numbers are given in brackets as follows: length of femur (5), length of tibia (6), metatarus length (7), metatarsus width (8), length of tergite 3 (9), length of tergite 4 (10), length of sternite 3 (11), length of wax plate of sternite 3 (12) width of wax plate of sternite 3 (13), distance between wax plates, sternite 3 (14), length of sternite 6 (15), width of sternite 6 (16), forewing length (17), forewing width (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), and wing angle O26 (31).

2.3. Data analysis

Morphological data were analysed using multivariate ANOVA, principal component analysis and linear discriminant analysis. All 27 morphological characters of individual worker bees were used in the analyses. Wilks' lambda test was used to compare multivariate means between the experimental groups. Scheffé multiple comparisons were used to compare univariate means between the groups (Johnson and Wichern, 2002). All statistical analyses were performed using Statistica© (StatSoft, 2004).

3. RESULTS

3.1. Group comparisons

Multivariate ANOVA using 27 morphometric characters of individual bees was carried out to test for within group and among group differences. A significant difference was found among the groups (MANOVA: Wilks' lambda: $\Lambda = 0.0003$, $F_{108,475} = 28.6$, $P < 0.0001$). No significant differences were found between the colonies within each group (MANOVA Wilks lambda: *cerana* control (1): $\Lambda = 0.14$, $F_{27,2} = 0.5$, $P = 0.8677$; *cerana* test (2): $\Lambda = 0.064$, $F_{27,2} = 1.2$, $P = 0.5470$; *cerana* cell-test (3): $\Lambda = 0.06$, $F_{27,2} = 1.1$, $P = 0.5846$; *mellifera* control (4): $\Lambda = 0.01$, $F_{27,2} = 7.9$, $P = 0.1183$; *mellifera* cell-test (5): $\Lambda = 0.04$, $F_{27,2} = 1.9$, $P = 0.4099$). Hence the significant differences found among the groups were not confounded by colony differences. Univariate ANOVA results showed significant mean differences among the worker groups in all 27 morphometric characters ($P < 0.05$, see Tab. II).

3.2. Effects of cell size

3.2.1. A. cerana in A. mellifera cells

The mean percentage increase in the 16 size-related characters between *A. cerana* in *A. cerana* cells reared by *A. cerana* control group (1) and *A. cerana* in *A. mellifera* cells reared by *A. cerana* cell-test group (3) was 8.2%. In ANOVA Scheffé multiple comparisons, the cell-test (larger) group (3) differed significantly from the control group (1) in 16 characters (14 size-related and 2 angle characters, Tab. II).

3.2.2. A. mellifera in A. cerana cells

The mean percentage decrease in the 16 size-related characters between *A. mellifera* in *A. mellifera* cells reared by *A. mellifera* control group (4) and *A. mellifera* in *A. cerana* cells reared by *mellifera* cell-test group (5) was 3.5%. In ANOVA Scheffé multiple comparisons, the cell-test (smaller) group (5) differed significantly from the control group (4) in 9 characters (7 size-related and 2 angle characters, Tab. II).

3.3. Effects of mixed-species

The mean percentage increase in the 16 size-related characters between *A. cerana* reared in *A. mellifera*-sized cells and reared either by *A. cerana* (3) or *A. cerana*/*A. mellifera* mixed-species (2) nurse bees was 11.6%. In ANOVA Scheffé multiple comparisons, the *cerana* test group (2) differed significantly from the cell-test group (3) in 22 characters (13 size-related and 9 angle characters, Tab. II).

Morphometric data (using 27 characters of individual workers) were submitted to principal component analysis to ascertain the number of group clusters, and resulted in five principal components, with eigenvalues greater than one, which represented 72.0% of the variation. PC1, correlated strongly ($|r| > 0.5$) with characters fem (5), tib (6), ltar (7), wtar (8), lt3 (9), lt4 (10), lst3 (11), lwm (12), wwm (13), lst6 (15), wst6 (16), lfw (17), wfw (18), cubital vein b (20), E9 (24), G18 (25), I10 (26), I16 (27), and N23 (30) (accounted for 45.0%), PC2 correlated strongly with cubital vein a (19), A4 (21), and O26 (31) (accounted for 8.7%), and PC3 with cubital vein a (19) and B4 (22) (accounted for 8.1%). The graph of principal components 1 and 2 scores revealed three distinct clusters: (A) *cerana* control group (1); (B) cell-test (larger) group (3); (C) *cerana* test (mixed nursing + large-cell) group (2), *mellifera* control group (4), and cell-test (smaller) group (5), (Fig. 1). The graphs of principal components 1 and 3 scores and 1 and 4 revealed very similar clusters.

A stepwise linear discriminant analysis using 27 morphometric characters of individual workers confirmed the separation of the three groups with 100% correct classification in the (A) *cerana* control group (1); 100% correct classification in the (B) cell-test (larger) group (3) and 100% correct classification in the (C) *cerana* test (mixed nursing + large-cell) group (2); *mellifera* control group (4)

Table II. Means and s.d. for 27 morphometric characters of individual *A. cerana* and *A. mellifera* workers bees by groups.

Character	(1) *A. cerana* control		(2) *A. cerana* test (mixed rearing + large-cell)		(3) *A. cerana* cell-test (larger)		(4) *A. mellifera* control		(5) *A. mellifera* cell-test (smaller)		ANOVA results
	mean	sd	mean	sd	mean	sd	mean	sd	mean	sd	significance
5. Femur	2.42^{a}	0.05	2.62^{b}	0.05	2.51^{c}	0.05	2.62^{b}	0.05	2.56^{c}	0.08	**
6. Tibia	3.03^{a}	0.08	3.17^{b}	0.09	3.16^{b}	0.06	3.15^{b}	0.08	3.15^{b}	0.08	**
7. ltar	1.91^{a}	0.04	2.07^{b}	0.06	2.00^{c}	0.04	2.03^{bc}	0.06	1.99^{c}	0.06	**
8. wtar	1.05^{a}	0.04	1.16^{b}	0.04	1.09^{c}	0.03	1.16^{b}	0.04	1.11^{c}	0.03	**
9. lt3	1.86^{a}	0.05	2.24^{b}	0.07	2.05^{c}	0.05	2.24^{b}	0.05	2.24^{b}	0.08	**
10. lt4	1.82^{a}	0.05	2.22^{b}	0.08	1.99^{c}	0.06	2.22^{b}	0.07	2.15^{d}	0.06	**
11. lst3	2.30^{a}	0.06	2.74^{b}	0.09	2.56^{c}	0.05	2.72^{b}	0.09	2.71^{b}	0.09	**
12. lwm	1.03^{a}	0.06	1.36^{b}	0.07	1.17^{c}	0.05	1.30^{d}	0.08	1.36^{b}	0.06	**
13. wwm	2.06^{a}	0.06	2.35^{b}	0.07	2.20^{c}	0.04	2.32^{bd}	0.09	2.28^{d}	0.06	**
14. Dwn	0.31^{a}	0.04	0.28^{ab}	0.03	0.22^{c}	0.03	0.26^{b}	0.04	0.21^{c}	0.03	**
15. lst6	2.24^{a}	0.06	2.58^{b}	0.09	2.42^{c}	0.06	2.56^{b}	0.07	2.60^{b}	0.09	**
16. wst6	2.81^{a}	0.05	3.22^{b}	0.15	2.86^{a}	0.07	3.11^{c}	0.09	3.05^{c}	0.16	**
17. lfw	8.62^{a}	0.11	9.19^{bd}	0.15	8.96^{c}	0.12	9.29^{b}	0.15	9.15^{d}	0.16	**
18. wfw	3.04^{a}	0.06	3.19^{b}	0.07	3.21^{bd}	0.05	3.33^{c}	0.07	3.26^{d}	0.09	**
19. cub1	0.550^{ab}	0.04	0.558^{ab}	0.05	0.579^{b}	0.03	0.553^{ab}	0.05	0.542^{a}	0.05	*
20. cub2	0.131^{a}	0.02	0.208^{b}	0.03	0.122^{a}	0.02	0.209^{b}	0.03	0.207^{b}	0.02	**
21. A4	31.08^{a}	1.79	32.90^{b}	1.96	31.68^{ab}	1.27	32.30^{ab}	2.32	31.17^{a}	2.29	**
22. B4	111.55^{ac}	3.73	105.00^{b}	5.56	111.27^{ac}	3.35	109.60^{a}	4.12	113.65^{c}	4.31	**
23. D7	94.78^{a}	5.44	99.41^{b}	4.41	93.72^{a}	3.27	99.63^{b}	6.08	99.33^{b}	2.39	**
24. E9	20.14^{a}	1.08	23.09^{b}	1.36	20.89^{a}	1.47	23.18^{b}	1.64	25.46^{c}	1.86	**
25. G18	85.64^{a}	3.02	94.59^{b}	2.79	88.24^{c}	3.59	92.13^{d}	2.93	90.31^{cd}	2.68	**
26. I10	47.20^{a}	2.84	55.31^{b}	3.80	47.35^{a}	2.65	54.13^{b}	3.66	53.61^{b}	3.54	**
27. I16	99.40^{a}	2.77	94.68^{b}	6.12	101.34^{a}	2.72	90.91^{c}	3.21	88.78^{c}	3.50	**
28. K19	78.61^{a}	2.28	75.94^{b}	2.90	78.58^{a}	2.56	76.50^{ab}	2.72	75.28^{b}	2.30	**
29. L13	14.98^{a}	1.35	14.15^{ab}	1.59	13.92^{b}	1.10	14.91^{a}	1.35	14.85^{ab}	1.54	**
30. N23	80.63^{a}	3.57	92.21^{b}	6.28	80.23^{a}	4.20	90.75^{b}	2.92	89.73^{b}	3.55	**
31. O26	34.92^{ac}	3.46	40.01^{b}	4.06	32.45^{a}	3.69	37.44^{bc}	3.90	35.54^{ac}	4.78	**

** $P < 0.01$, * $P < 0.05$; Different letters in rows indicate that means are significantly different at the 0.05 level.

and cell-test (smaller) group (5). Listed are the 11 characters selected by the stepwise procedure and ranked according to their discriminatory power: lt3 (9), cubital vein b (20), lst3 (11), lst6 (15), N23 (30), I16 (27), dwn (14), lt4 (10), wwm (13), wfw (18) and tib (6).

4. DISCUSSION

The results clearly show three significantly different and well-defined morphoclusters of worker honeybees obtained under varying conditions of nutrition and cell size. It is

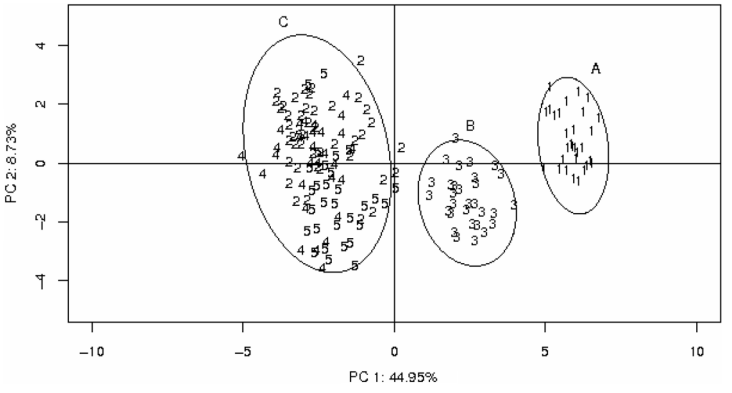

Figure 1. The graph of principal components using 27 morphometric characters of individual bees revealed three clusters: (A) *A. cerana* control group (1); (B) cell-test (larger) group (3); (C) *A. cerana* test (mixed rearing + large-cell) group (2); *A. mellifera* control group (4) and cell-test (smaller) group (5). 95% confidence ellipses.

therefore essential to attempt a partitioning of the two variables, nutrition and cell size, to assess their individual effects. That changes in cell size results in changes in honeybee size dimensions are supported in the following comparisons: (1) those *A. cerana* reared in *A. mellifera* cells but reared only by *A. cerana* nestmates were significantly larger than the *A. cerana* control group (Tab. II). However, the *A. mellifera* cells are somewhat larger than those of *A. cerana* and in consequence this could have resulted in a greater amount of food having been supplied to larvae in the larger cells (Haydak, 1970), making this result somewhat equivocal; (2) against this, in the comparison of *A. mellifera* in *A. mellifera* cells reared by *A. mellifera* (*mellifera* control group) and *A. mellifera* in *A. cerana* cells reared by *A. mellifera* (*mellifera* small cell-test group) the groups were morphologically indistinguishable in half of the dimensional characters for size (Tab. II). The results obtained from *A. mellifera* bees reared in *A. mellifera*-sized and *A. cerana*-sized cells is the converse experiment where nursing bees again remains a constant for quality and only cell size varied. Here, those *A. mellifera* reared in the *A. cerana*-sized cells were significantly smaller in size for 9 characters than those reared in normal size *A. mellifera* cells (Tab. II).

The comparisons of greatest interest are those for *A. cerana* reared in *A. mellifera*-sized cells and reared either by *A. cerana* or *A. cerana*/*A. mellifera* mixed-species nurse bees. Here, cell size is a constant and nurse bees the only variable. In this case, the bees from the mixed-species feeding regime were significantly larger in 81% of characters than those bees reared in *A. mellifera*-sized cells (Tab. II). The mean percentage increase was 10.6% for all characters and 11.6% for size characters, the latter equates to an average size increase for all characters of 1.5 mm. The results unequivocally establish that the presence of *A. mellifera* more greatly enhance growth of *A. cerana* larvae than does that of their own nurse bee sisters. This effect is independent of the developmental plasticity in changing morphological characters.

The pressing question now is how are the mixed-species rearing effects to be interpreted. Because we have photographed heterospecific trophallaxis, it is inferred that these changes in *A. cerana* workers reared in mixed-species colonies can be attributed to heterospecific feeding and/or differences in quality or quantity of royal jelly proffered by nurse bees, just as obtained in rearing mega-queens (Tan et al., 2006). Admittedly, we do not know the relative contributions of the two species of nurse bees in the mixed-species colonies. Nonetheless, the only remaining variables are possible differences in the relative amounts and/or quality of royal jelly that are given

the developing larvae by *A. cerana* and *A. mellifera* nurse bees. Although the royal jelly of *A. cerana* contains a higher ratio of protein to carbohydrate than that of *A. mellifera,* the latter produces a greater volume/worker (Takenaka and Takenaka, 1996). Even within *A. mellifera*, highly bred strains such as *A. m. carnica* produce more royal jelly than their genetically more variable but genetically unselected Africanized counterparts. It is nonetheless clear that provision of *A. mellifera* royal jelly to *A. cerana* worker larvae results in worker gigantism just as it leads to queen gigantism. However it remains quite uncertain whether these results derive from quantity or quality.

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Effets induits par l'environnement sur les caractéristiques morphométriques des ouvrières dans les colonies d'*Apis cerana*.

***Apis cerana* / *Apis mellifera* / morphométrie / développement postembryonnaire / taille de la cellule / colonie composée de plusieurs espèces.**

Zusammenfassung – Umweltinduzierte Einflüsse auf die morphometrischen Eigenschaften von Arbeiterinnen in Völkern von *Apis cerana*. Wir untersuchten den Einfluss von Völkern, die entweder nur *Apis cerana* oder nur *A. mellifera* oder eine Mischung dieser beiden Arten enthielten sowie den Einfluss unterschiedlicher Zellgrößen auf die Entwicklung der morphologischen Eigenschaften von *A. cerana* Arbeiterinnen. Es wurden Bienen aus 5 experimentellen Gruppen untersucht (vergl. Tab. I). Eine grafische Darstellung der Hauptfaktoren zeigte drei getrennte morphologische Kluster: (A) von *A. cerana* Arbeiterinnen in *A. cerana* Zellen aufgezogene *A. cerana* (*Cerana* Kontrollgruppe); (B) von *A. cerana* Arbeiterinnen in *A. mellifera* Zellen aufgezogene *A. cerana* (*Cerana* Zellentestgruppe); (C) von gemischten *A. cerana* und *A. mellifera* Arbeiterinnen in *A. mellifera* Zellen aufgezogene *A. cerana* (*Cerana* Testgruppe) zusammen mit von *A. mellifera* Arbeiterinnen in *A. mellifera* Zellen aufgezogenen *A. mellifera* (*Mellifera* Kontrollgruppe) und von *A. mellifera* in *A. cerana* Zellen aufgezogenen *A. mellifera* (*Mellifera* Zellentestgruppe). Übernormal große *A. cerana* Arbeiterinnen können sowohl durch die Aufzucht in größeren Zellen als auch in Völkern mit gemischter Artzusammensetzung erzeugt werden. Unterschiede innerhalb und zwischen den Gruppen wurden mit multivariater ANOVA untersucht, zwischen den Völkern innerhalb der Gruppen bestanden keine signifikanten Unterschiede. (Tab. II).
Der mittlere prozentuale Zuwachs der 16 größenbezogenen Charaktere zwischen der *A. cerana* Kontrollgruppe (1) und der Zellentestgruppe (3) betrug 8,2 %. In den ANOVA Scheffé Mehrfachvergleichen unterschieden sich diese signifikant (Tab. II). Die mittlere prozentuale Abnahme für die *A. mellifera* Kontrollgruppe (4) und Zellentestgruppe (5) betrug 3,5 %. Der Zuwachs zwischen in Mellifera-Zellen aufgezogenen *A. cerana* die entweder von *A. cerana* (3) oder aus *A. cerana* und *A. mellifera* gemischten Ammenbienen aufgezogen wurden (2) betrug 11,6 %.
Dass die Zellengrösse allein die Größendimensionen der Bienen ändern kann zeigt sich darin, dass die von ausschließlich *A. cerana* Arbeiterinnen in *A. mellifera* Zellen aufgezogenen *A. cerana* größer waren als die *A. cerana* Kontrollgruppe. Bei den Vergleichen zwischen den in *A. mellifera* Zellen entweder von *A. cerana* oder aus *A. cerana* und *A. mellifera* zusammengesetzten Völkern aufgezogenen *A. cerana* ist die Zellengröße konstant und die Ammenbienen sind die einzige Variable. In diesem Falle waren die den Völkern mit gemischter Artzusammensetzung entstammenden Bienen signifikant größer als die in *A. mellifera* großen Zellen aufgezogenen Bienen (Tab. II). Dieser Versuch zeigt, dass das Wachstum von *A. cerana* Larven durch die Anwesenheit von *A. mellifera* besser gefördert wird als durch ihre eigenen Ammenschwestern. Wir leiten daraus ab, dass diese bei *A. cerana* bewirkten Änderungen auf heterospezifische Fütterung in den gemischt zusammengesetzten Völkern und Unterschieden in der Qualität oder Quantität des Futtersaftes der Ammenbienen bewirkt wird.

***Apis cerana* / *Apis mellifera* / Morphometrie / Entwicklung / Völker mit gemischter Artenzusammensetzung**

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