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ORIGINAL ARTICLE

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Gigantism in honeybees: *Apis cerana* queens reared in mixed-species colonies

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Abstract The development of animals depends on both genetic and environmental effects to a varying extent. Their relative influences can be evaluated in the social insects by raising the intracolonial diversity to an extreme in nests consisting of workers from more than one species. In this study, we studied the effects of mixed honeybee colonies of *Apis mellifera* and *Apis cerana* on the rearing of grafted queen larvae of *A. cerana*. *A. mellifera* sealed worker brood was introduced into *A. cerana* colonies and on emergence, the adults were accepted. Then, *A. cerana* larvae were grafted for queen rearing into two of these mixed-species colonies. Similarly, *A. cerana* larvae and *A. mellifera* larvae were also grafted conspecifically as controls. The success rate of *A. cerana* queen rearing in the test colonies was 64.5%, surpassing all previous attempts at interspecific queen rearing. After emergence, all virgin queens obtained from the three groups (N=90) were measured morphometrically. The *A. cerana* queens from the mixed-species colonies differed significantly in size and pigmentation from the *A. cerana* control queens and closely approximated the *A. mellifera* queens. It is inferred that these changes in the *A. cerana* queens reared in the mixed-species colonies can be attributed to feeding by heterospecific nurse bees and/or chemical differences in royal jelly. Our data show a strong impact of environment on the development of queens. The results further suggest that in honeybees the cues for brood recognition can be learned by heterospecific workers after eclosion, thereby providing a novel analogy to slave making in ants.

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Introduction

Species-specific conditions for the development of social insects include genetic and environmental differences such as pheromones, feeding and rearing regimes, worker behavior, and queen status. The relative importance of genetics or environment on development can be evaluated in social insects by increasing the intracolonial environmental diversity to an extreme using workers from different species. This naturally occurs in slave-making ants (Hölldobler and Wilson 1990), but can also be experimentally achieved in honeybees.

While honeybee workers and queens can be reared in any intraspecific colony, interspecific reciprocal introductions of female larvae between *A. mellifera* and *A. cerana* have usually failed (Oschmann 1965; Dhaliwal and Atwal 1970; Adlakha and Sharma 1971; Oku and Ono 1990; Potichot et al. 1993). The workers may possibly reject such foreign larvae because of species-specific brood pheromones (Potichot et al. 1993; Ayasse and Paxton 2002) and/or differences in royal jelly (Inoue 1962; Takenaka and Takenaka 1996; Su et al. 2005). In contrast to brood, young adult workers of *A. mellifera* are readily accepted in *A. cerana* colonies and vice versa (Atwal and Sharma 1967; Dhaliwal and Atwal 1970), probably because they have not yet developed recognition cues (Breed et al. 2004).

If recognition cues for brood are species-specific in honeybees and not learned after eclosion, then heterospecific introductions of workers may result in either the rejection of introduced larvae or in reduced overall feeding. If, however, cues can be learned by heterospecific workers

after eclosion, then larvae introduced into mixed colonies would be expected not to differ from controls, or could actually become larger than intraspecific *A. cerana* controls because of the higher potential feeding rate of *A. mellifera* (Oku and Ono 1990) and species-specific differences in the royal jelly composition (Takenaka and Takenaka 1996). We chose a queen model because such larvae are more intensively nurtured and scrutinized than worker larvae by honeybees (Kuwabara 1947; Ribbands 1953; Takenaka and Takenaka 1996). In this study, we test whether mixed-species colonies of *A. cerana* and *A. mellifera* could appreciably alter heterospecific transfer barriers of larval queen grafts, result in a different larval acceptance and adult emergence rate as well as in changes in morphological characters in such queens.

Materials and methods

Experiments

Experiments were performed at the apiary of Yunnan Agricultural University. Two frames of sealed worker brood (~1,500 cells) of *A. mellifera* were introduced into each of the four-queenright *A. cerana* colonies to achieve mixed-species environments. Each colony had a young, naturally mated, laying queen and three frames of *A. cerana* brood and workers. When all the *A. mellifera* sealed brood emerged, two of the four mixed-species colonies were dequeened to serve as test colonies for the introduction of 62 1-day-old *A. cerana* grafted larvae from the four original colonies. In these two queenless mixed-species test colonies, the *A. cerana* larvae were exposed to both *A. mellifera* and *A. cerana* nurse-age worker cohorts (group 1: *A. cerana* test). In two other dequeened *A. cerana* colonies, 64 1-day-old grafted larvae of *A. cerana*, introduced for queen rearing, were only exposed to conspecific nurse bees (group 2: *A. cerana* control). Similarly, 76 *A. mellifera* 1-day old grafted larvae were introduced in two dequeened *A. mellifera* colonies (group 3: *A. mellifera* control). The acceptance of larvae for queen rearing was evaluated in all test and control colonies. On emergence, 30 virgin queens were collected from each of the three groups for subsequent measurements.

Measurements

To evaluate the possible effects of the mixed species colonies on queen rearing, measurements on newly emerged queens were performed using a stereo-microscope and a computer-aided measuring system based on a video system and measuring program (Meixner 1994). The queens were measured according to the methods of Ruttner et al. (1978) and Ruttner (1988) and their Ruttner (1988) numbers are given in parentheses. Thirty-one characters of queens (seven pigmentation and 24 metrical) were measured: length of femur (5), length of tibia (6), metatarsus length (7), metatarsus width (8), tergite 3 longitudinal (9), tergite 4, longitudinal (10), sternite 6, longitudinal (15), sternite 6, transverse (16), forewing, longitudinal (17), forewing, transverse (18), cubital vein distance a (19), cubital vein distance b (20), wing angles A4 (21), B4 (22), D7 (23), E9 (24), G18 (25), I10 (26), I16 (27), K19 (28), L13 (29), N23 (30), O26 (31), and hamuli. The pigmentation of tergite 2 (32), tergite 3 (33), tergite 4 (34), scutellum (35), scutellar plate (36), labrum (lab 1), and clypeus (lab 2) were graded from 0 to 9 according to Ruttner (1988).

Data analyses

Data were analyzed using multivariate ANOVA, principal components analysis, and linear discriminant analysis. Wilks' lambda test was used to compare multivariate means between groups. Scheffe's multiple comparisons were used to compare univariate means between groups. The Mahalanobis squared distances (d^2) between the groups were calculated separately (Johnson and Wichern 1998). Queen rearing success rates for one colony of each group were analyzed using Chi-square tests of independence.

Results

Acceptance of adult *A. mellifera* workers and queen-rearing success rates for *A. cerana*

The *A. mellifera* workers that emerged over a 3-day period were accepted. Neither fighting was seen on opening the four mixed-species colonies nor were dead *A. mellifera* workers found at the entrances. During these colony inspections, the *A. mellifera* workers were observed entering the *A. cerana* queen cups. The results of the introductions of the larval grafts into one colony of each group were as follows: (1) of 62 *A. cerana* larvae grafted in the two test colonies, 51 were accepted and 40 queens emerged as adults resulting in a success rate of 64.5%; (2) in the *A. cerana* control colonies, 64 *A. cerana* larvae were grafted, 52 were accepted and 45 emerged as adult queens, a success rate of 70.3%; (3) in the *A. mellifera* control colonies, 76 larvae were grafted, 70 were accepted and 67 emerged as adult queens, a success rate of 88.2%. The data show that there were no significant differences in the relative acceptance rates among the *A. cerana* test and control groups (Chi-square test χ^2=0.48, *df*=2, *P*=0.488). However, a significantly higher acceptance rate was found in *A. mellifera* compared to the *A. cerana* test group (Chi-square test χ^2=20.8, *df*=2, *P*<0.001). The acceptance rates between the *A. mellifera* and *A. cerana* control groups were also significantly different (Chi-square test χ^2=15.7, *df*=2, *P*<0.001).

Morphometrics of newly emerged queens

Univariate two-way ANOVA tests, with groups and colonies as factors, showed that the three groups of queens, *A. cerana* test and control, and *A. mellifera* control queens differed significantly for all seven color characters (Table 1), while there were no significant differences between colonies for each group (MANOVA: Groups: Wilks' lambda=0.021, $F_{14,156}$=66.1, P<0.0001; Colonies: Wilks' lambda=0.972, $F_{7,78}$=0.32, P=0.9416). The newly emerged *A. cerana* test queens reared in the mixed-species colonies were intermediate between the dark brown control queens of *A. cerana* and the very yellow ones of *A. mellifera*, but closer in color to the latter (Table 1). The *A. cerana* control and test groups differed significantly for five characteristics of color; *A. cerana* test queens differed from the *A. mellifera* control queens for seven and, the *A. cerana* test queens and *A. mellifera* control queens for seven color characters (Scheffe's multiple comparison tests, Table 1).

The morphometric measurement data for 24 characters showed significant mean differences for all three queen groups (Table 2), while no significant differences were found between colonies for each group (MANOVA: Groups: Wilks' lambda=0.002, $F_{48,122}$=59.7, P<0.0001; Colonies: Wilks' lambda=0.745, $F_{24,61}$=0.89, P=0.6134). Univariate two-way ANOVA results showed significant mean differences between the groups for 21 morphometric characters (P<0.05, see Table 2). In Scheffe's multiple comparisons, the *A. cerana* test group differed significantly in 18 characters from the *A. cerana* control group, and in 15 from the *A. mellifera* control group. The *A. mellifera* control queens differed from the *A. cerana* control group in 15 characters.

Morphometric data (using seven pigmentation and 24 metrical characters) were analyzed by a principal components analysis, resulting in seven principal components, with eigen values greater than one, which represented 74.3% of the variation. PC1, correlated strongly ($|r|$>0.5) with characters (5–10, 15–17, 26, 30–34, 35, 36), labrum and hamuli accounting for 40.7%, PC2 correlated strongly with (20, 22, 23, 26) accounting for 10.8%, and PC3 mainly with (19) accounting for 6.0%. The graph of principal components 1 and 2 scores revealed three groups: the *A. cerana* queen control group differed significantly from the other groups, while the *A. cerana* test group and *A. mellifera* group were closely neighboring each other (Fig. 1). A stepwise linear discriminant analysis using all 31 morphometric characters confirmed the separation of the groups with 100% correct classification in each group. The 15 principal characters ranked according to their discriminatory power were: (15), (33), (22), (31), (16), (17), labrum, (20), (21), clypeus, (35), (27), (9), (24), and (7). The Mahalanobis squared distances between the centroids of the groups are 334.1 between *A. cerana* test and *A. cerana* control groups; 55.7 between *A. cerana* test and *A. mellifera* control, and 281.1 between *A. cerana* control and *A. mellifera* control groups (Fig. 2.).

Discussion

Our data confirm that young *A. mellifera* workers can be accepted in *A. cerana* host colonies (Atwal and Sharma 1967; Dhaliwal and Atwal 1970) because the workers emerging from *A. mellifera* brood frames within the *A. cerana* host colonies were adopted. This happened probably without aggression because neither fighting was seen on opening the hives nor were dead *A. mellifera* workers found at the entrances. Thus, it is possible to overcome heterospecific acceptance barriers against adults by using emerging *A. mellifera* workers in queenright *A. cerana* host colonies. Such young honeybee workers lack olfactory sensitivity (Pham-Delegue et al. 1993; Laloi et al. 2001) and behavioral aggressiveness and cannot participate in pheromonally induced colony defense (Whiffler et al. 1988). They are also in the nurse-bee stage of division of labor and may be expected to participate in heterospecific brood rearing unless they discriminate the grafted *A. cerana* larvae as artifacts. Therefore, our mixed-species colony approach enables to expose *A. cerana* brood to a brood-rearing environment influenced by the presence of *A. mellifera* workers.

While only three virgin queens were successfully produced in one experiment (Inoue 1962), all other attempts to raise queens from heterospecific grafts of *A. cerana* and *A. mellifera* completely failed (Oschmann 1965; Dhaliwal and Atwal 1970; Oku and Ono 1990;

Table 1 Graded pigmentation for *A. cerana* test queens reared in the mixed species colonies (N=30), *A. cerana* control queens (N=30), and *A. mellifera* control queens (N=30)

Character	*A. cerana* test		*A. cerana* control		*A. mellifera* control		ANOVA results	
	Mean	SD	Mean	SD	Mean	SD	F-statistic	P-value
Tergite 2	7.70[a]	1.15	5.37[b]	0.49	9.00[c]	0.00	$F_{2,84}$=197.1	<0.0001
Tergite 3	8.33[a]	0.48	5.60[b]	0.81	9.00[c]	0.00	$F_{2,84}$=319.5	<0.0001
Tergite 4	7.67[a]	0.99	5.90[b]	0.88	8.80[c]	0.41	$F_{2,84}$=98.5	<0.0001
Scutellum	2.13[a]	0.57	1.37[a]	1.16	4.03[c]	2.50	$F_{2,84}$=20.8	<0.0001
Scutellar plate	4.17[a]	1.15	1.77[b]	1.01	5.63[c]	0.81	$F_{2,84}$=111.8	<0.0001
Labrum	2.60[a]	0.77	6.53[b]	0.51	4.50[c]	1.61	$F_{2,84}$=99.2	<0.0001
Clypeus	4.40[a]	2.06	3.83[a]	1.42	2.63[c]	1.54	$F_{2,84}$=8.3	0.0005

Means and standard deviations are shown. Different letters indicate that means are significantly different at the 0.05 level

Table 2 Metrical characters of *A. cerana* test queens reared in the mixed species colonies (*N*=30), *A. cerana* control queens (*N*=30), and *A. mellifera* control queens (*N*=30)

Character	*A. cerana* test		*A. cerana* control		*A. mellifera* control		ANOVA results	
	Mean	SD	Mean	SD	Mean	SD	*F*-statistic	*P*-value
5	325.25[a]	4.77	302.52[b]	9.93	332.18[c]	13.67	$F_{2,84}$=67.9	<0.0001
6	365.74[a]	11.53	350.39[b]	11.42	379.21[c]	7.13	$F_{2,84}$=59.9	<0.0001
7	245.79[a]	9.18	225.45[b]	12.91	261.58[c]	8.86	$F_{2,84}$=88.5	<0.0001
8	124.02[a]	3.65	112.93[b]	8.34	124.94[a]	4.95	$F_{2,84}$=42.5	<0.0001
9	318.04[a]	9.04	288.74[b]	13.38	334.68[c]	11.11	$F_{2,84}$=137.1	<0.0001
10	311.79[a]	7.52	283.09[b]	8.46	324.62[c]	9.64	$F_{2,84}$=186.1	<0.0001
15	364.39[a]	10.90	296.83[b]	7.22	357.10[c]	8.67	$F_{2,84}$=495.5	<0.0001
16	365.32[a]	11.86	306.36[b]	15.47	386.92[c]	17.62	$F_{2,84}$=227.6	<0.0001
17	1,014.87[a]	10.62	1,005.14[b]	11.01	1,037.48[c]	12.07	$F_{2,84}$=63.4	<0.0001
18	347.79[a]	6.71	345.50[a]	8.32	354.00[c]	7.44	$F_{2,84}$=11.5	<0.0001
19	63.56[a]	5.97	65.48[a]	3.82	63.75[a]	5.01	$F_{2,84}$=1.3	0.2762
20	21.42[a]	2.49	16.26[b]	2.58	17.58[b]	1.96	$F_{2,84}$=38.6	<0.0001
21 = A4	38.87[a]	1.80	38.38[ab]	3.06	37.31[b]	1.42	$F_{2,84}$=3.8	0.0257
22 = B4	87.15[a]	3.04	95.40[b]	4.25	97.73[b]	4.48	$F_{2,84}$=57.7	<0.0001
23 = D7	99.29[a]	4.49	95.77[b]	3.52	95.47[b]	3.01	$F_{2,84}$=10.0	<0.0001
24 = E9	20.41[a]	1.22	22.00[b]	2.44	21.44[ab]	1.70	$F_{2,84}$=5.5	0.0057
25 = G18	99.48[a]	9.53	100.21[a]	4.43	100.79[a]	6.54	$F_{2,84}$=0.3	0.7821
26 = I10	51.42[a]	3.49	42.07[b]	3.23	45.57[c]	2.35	$F_{2,84}$=72.2	<0.0001
27 = I16	95.98[a]	2.26	96.41[a]	3.28	94.47[a]	4.63	$F_{2,84}$=2.5	0.0855
28 = K19	76.36[a]	3.27	74.06[b]	3.99	78.28[a]	2.05	$F_{2,84}$=13.1	<0.0001
29 = L13	15.64[a]	1.27	14.74[ab]	2.33	14.07[b]	1.47	$F_{2,84}$=6.2	0.0031
30 = N23	84.48[a]	4.29	74.61[b]	3.47	86.30[a]	3.24	$F_{2,84}$=85.1	<0.0001
31 = O26	33.76[a]	3.60	21.10[b]	2.53	32.62[a]	3.68	$F_{2,84}$=131.5	<0.0001
Hamuli	19.43[a]	1.48	17.50[b]	1.28	19.60[a]	1.45	$F_{2,84}$=20.4	<0.0001

Means and standard deviations are shown. Different letters indicate that means are significantly different at the 0.05 level

Potichot et al. 1993). In those experiments, the authors used single-species host colonies. However, in our experiments, we specifically chose to graft *A. cerana* larvae into mixed-species colonies to at least partly overcome the barrier in cross-fostered brood-rearing between *A. cerana* and *A. mellifera*. Indeed, the acceptance of *A. cerana* queens in a mixed-species colony was not significantly different from the *A. cerana* control, suggesting that the introduced *A.*

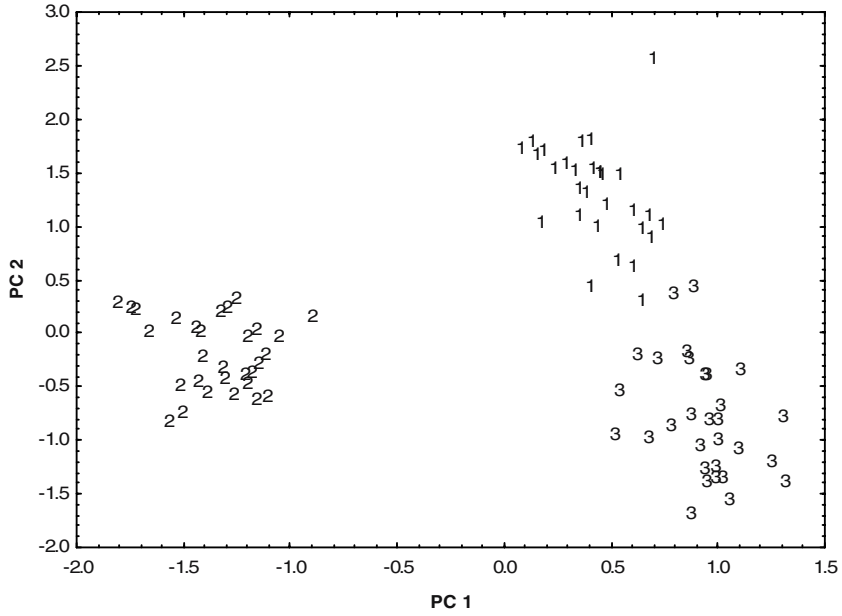

Fig. 1 The graph of principal components 1 and 2 scores showing groups based on seven pigmentation and 24 metrical characters of queens (*1A. cerana* test group, *2A. cerana* control group; *3A. mellifera* control)

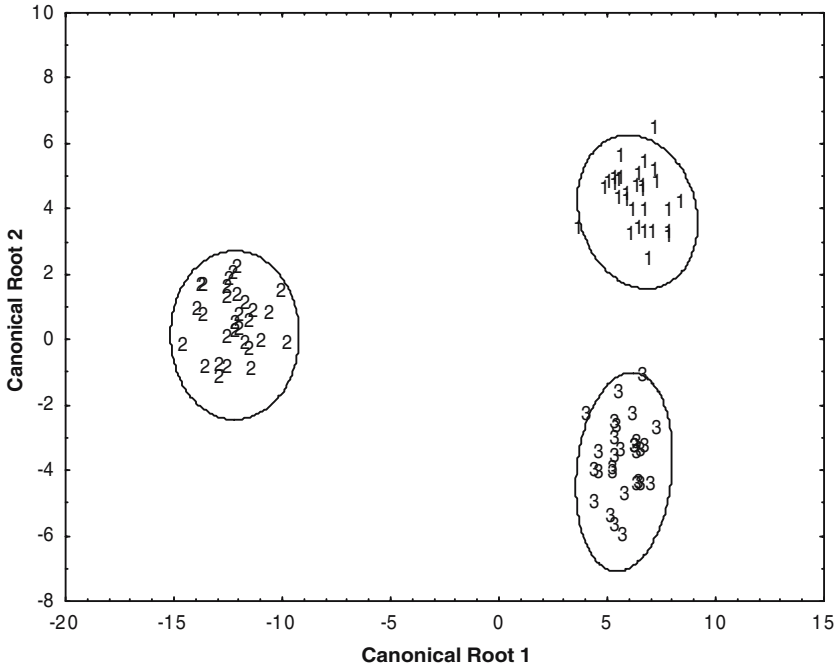

Fig. 2 The graph of canonical scores 1 and 2 showing 95% confidence ellipses for the three groups using 11 principal morphometric characters ranked according to their discriminatory power: (15), (33), (22), (31), (16), (17), labrum, (20), (21), clypeus, (35), (27), (9), (24), and (7) (see "Materials and methods" section for details); (*1A. cerana* test group, *2A. cerana* control group; *3A. mellifera* control)

mellifera workers did not discriminate against the grafted *A. cerana* larvae.

That the newly emerged *A. cerana* test queens were intermediate in color between the dark brown *A. cerana* controls and the very yellow *A. mellifera* counterparts was an unexpected result. Coloration is a variable of interest in development because phenotypic expression is temperature sensitive (Tsurata et al. 1989) and there are also seasonal differences in some strains of *A. cerana* (Matsuka et al. 1995). The mixed species composition of nurse bees might also have influenced worker behavior, hence temperature in the brood nest. However, in our experiments, the same strain of *A. cerana* was used over the same time period and it is well established that the average brood nest temperature under the same field conditions is virtually the same in both *A. cerana* and *A. mellifera* (Yang 2001). Although temperature was not measured in this experiment, it remains a reasonable assumption that temperature will be the same in mixed-species colonies so that variations in temperature are considered as systematic errors in our experiment. The only other obvious remaining variable is the brood-rearing environment provided by the two species of nurse bees. The quality and quantity of royal jelly is one aspect. The gross chemical composition of royal jelly of *A. cerana* and *A. mellifera* is known to differ and royal jelly is a rich source of numerous different carotenoids (Takenaka and Takenaka 1996; Su et al. 2005). Although no experiments have been reported on the effects of royal jelly in relation to pigmentation, it is reasonable to argue by analogy to other animals such as aquarium fish and caged song birds that a greater intake of carotenoids supplied by the *A. mellifera* nurse bees could well explain the intermediate coloration of the test queens.

The morphometric data showed significant differences between the queen groups. Indeed, the *A. cerana* test queens were 11.1% larger than the *A. cerana* controls and only 4.6% smaller than those of *A. mellifera.* That such exceptionally large queens of *A. cerana* were produced in these experiments may be attributed to different feeding of the nascent queen larvae by the mixed-species colonies, e.g., due to the higher potential feeding rate of *A. mellifera* workers (Oku and Ono 1990), and/or to the differences in royal jelly quality between the two species (Takenaka and Takenaka 1996). Therefore, the significant morphometric differences between the *A. cerana* test and control queens may also indicate that the *A. mellifera* workers have probably participated in rearing the heterospecific larvae. This seems to imply that the cues used by *A. mellifera* nurse-age bees for brood recognition can be learned after eclosion even if they are derived from another species. Given that the *A. mellifera* workers actually participated in heterospecific brood rearing in the *A. cerana* host colonies, this would constitute an analogy to slave-making ants, where workers of the slave species participate in brood rearing of the slave-making one (Hölldobler and Wilson 1990). Such learning of recognition cues might be adaptive in a general honeybee context because it naturally accommodates the production of queens during supersedure or in emergency queen rearing. Any new queen might provide slightly different olfactory cues for its offspring and it is essential for colony survival that all brood derived from a new queen is readily accepted by newly emerging nurse bees.

In conclusion, our data confirm that young *A. mellifera* workers can be accepted in *A. cerana* colonies (Atwal and Sharma 1967; Dhaliwal and Atwal 1970). *A. cerana* queens can be reared in such mixed-species colonies of *A. cerana* and *A. mellifera* just as successfully as in an *A. cerana* colony, suggesting that introduced *A. mellifera* workers may participate in rearing heterospecific larvae.

The *A. cerana* queens produced in this way showed significant differences in both pigmentation and morphometric values compared to their conspecific controls demonstrating a strong environmental impact on development. We suggest the use of our mixed-species colony approach in future studies, which may lead to a better understanding on the influence of genetics and environment on the development of social insects.

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