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Correlations between morphology and colony defence in *Apis mellifera* L.[1]

ANITA M COLLINS[2,4]; HOWELL V DALY[3]; THOMAS E RINDERER[2]; JOHN R HARBO[2]; KIM HOELMER[5]

[2]USDA, ARS, Honey Bee Breeding, Genetics and Physiology Laboratory, 1157 Ben Hur Road, Baton Rouge, LA 70820, USA

[3]Department of Entomological Sciences, University of California, Berkeley, CA 94720, USA

[5]USDA, APHIS, PPQ, Phoenix Plant Methods Center, c/o Irrigated Desert Research Station, 4151 Highway 86, Brawley, CA 92227, USA

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SUMMARY

Significant correlations between 25 quantitative characters of worker honey bees used for the morphometric identification of Africanized honey bees (*Apis mellifera*), seven measures of colony defence and 12 for alarm pheromone production were calculated from data on colonies in Louisiana, USA, and Monagas, Venezuela, two years after the arrival of Africanized honey bees in the eastern portion of Venezuela. The bees in the Venezuela group were identified as European (70%), European with evidence of introgression of Africanized genes (5%), Africanized with evidence of introgression of European genes (7%) and Africanized (18%), indicative of a population undergoing hybridization. For the Venezuelan population alone, the correlations between defensive behaviour and morphometric identification as Africanized were not significant. Therefore, defensive behaviour alone is not an adequate indicator for identification or certification programmes in areas undergoing Africanization.

Keywords: *Apis mellifera*, Africanized honey bees, honey bee colonies, worker honey bees, alarm pheromones, colony defence, morphometrics, hybridization, identification, Venezuela

[1]All editorial functions for this paper, including the selection of referees, have been undertaken by staff at IBRA headquarters

[4]Present address: USDA, ARS, SARL, Honey Bee Research Unit, 2413 East Highway 83, Weslaco, TX 78596, USA

INTRODUCTION

On 15 October 1990, the first swarm of Africanized honey bees (*Apis mellifera* L.) in the USA was discovered in Texas on the USA/Mexico border. One year later, eighteen counties of south Texas were under quarantine by the state regulatory agency due to the presence of this honey bee type (F Maxwell, Texas Apiary Inspection Service). A critical aspect of the monitoring process has been the ability to determine which honey bee swarms or colonies were of the Africanized type.

Distinguishing between the Africanized and European ecotypes of the Western honey bee has always been a concern. Currently, the only formally accepted method for the identification of Africanized/European honey bees is the morphometric system of Daly and Balling (1978) or its derivations, FABIS (Sylvester & Rinderer, 1987) and USDA-ID (Rinderer *et al.*, 1993). FABIS (Fast Africanized Bee Identification System) is designed to be used as a rapid screening technique to classify samples of 10–30 worker bees as European or unknown, using such measures as degastered body weight or forewing length. USDA-ID is an enhanced version of the sophisticated morphometric identification system of Daly and Balling using computer-assisted measurement of body parts from 10 workers. This system is based on over 2 000 different colony samples from throughout South, Central and North America, and Australia.

Because of the time and equipment required to identify colonies using morphometrics, it has been suggested that defensive behaviour may be an appropriate way to make judgements about a colony's genotype in the field. Levels of defensive behaviour are currently one way that the Texas Apiary Inspection Service identifies swarms or colonies that may be Africanized and require immediate processing (Chandler, 1993). However, only if significant correlations exist between morphometric measures and defensive behaviour can the vigour of a colony's defence alone be an appropriate way to distinguish Africanized honey bee colonies from European.

We examined the usefulness of colony defence as a field identification method using a database having both morphometric and colony defence measurements on 293 colonies ranging from commercial European to strongly Africanized-like. Phenotypic correlations between colony defensive behaviour and the morphology were calculated to evaluate the reliability of defence tests for identification.

MATERIALS AND METHODS

Data were compiled from a survey of honey bee colonies conducted in 1979 in Baton Rouge, Louisiana, USA, and in Maturin, Monagas, Venezuela. The sample collection was completed 2–3 years after the arrival of Africanized honey bees (AHB) to the Maturin area (Hellmich & Rinderer, 1991; Taylor & Levin, 1978). The 150 colonies in Louisiana had been established from various United States commercial honey bee stocks. The 147 Venezuelan colonies were either ones that had originally been headed by European queens but were chosen on the basis that they had not been requeened within at least the past year (unmanaged), or had been established from locally caught swarms (Taylor, 1979) within the same time frame (swarms). Some samples were damaged or lost, and only a portion of the Louisiana colonies were assayed for all pheromones. Actual values of n for all calculations are reported in the tables.

Samples of 10 worker bees from each colony in the survey were collected, placed in alcohol and identified by the morphometric procedure of Daly *et al.* (1982). For the present investigation, the original specimens were reclassified using the discriminant functions of Rinderer *et al.* (1993) for USDA-ID. This procedure classifies each sample as Africanized, Africanized with evidence of introgression of European genes, European with evidence of introgression of African genes, or European based on a probability of the sample of 10 bees being Africanized or European. European classified colonies are bees originating from temperate-evolved ecotypes (European subspecies and their derivatives currently represented in USA commercial stocks). Africanized colonies are bees of a tropical ecotype (South and Central American populations derived from *A. mellifera scutellata* from South Africa hybridized to some extent with European subspecies (Buco *et al.*, 1987; Ruttner, 1986; Sheppard *et al.*, 1991; Moritz & Meusel, 1992).

The results of tests of colony defence in the 1979 survey were reported in Collins *et al.* (1982). Each colony was tested twice in a standard, 90-s test sequence (Collins & Kubasek, 1982). Observations of defensive behaviour were quantified in four ways: (1) the number of bees at the colony entrance was counted from photographs taken at 30-s intervals during the test (pretest, and 30 s, 60 s and 90 s after initial stimulation of the colony with a component of honey bee alarm pheromone, 3 ml of 1% isopentyl acetate in paraffin oil); (2) the time until bees began to emerge from the colony after alarm pheromone was sprayed above the entrance; (3) the time for the first bee to land on one of two moving suede leather targets in front of the colony; and (4) the number of stings in the targets.

The chemical analysis of alarm pheromones present in three samples from each of the surveyed colonies was reported in Collins *et al.* (1989). Samples of bees from each colony were collected from the entrance in a plastic bag and killed by freezing. The sting apparatus and head of each bee were

TABLE 1. Morphometric classification of 150 colonies from Baton Rouge, LA, USA, and 143 colonies from Maturin, Monagas, Venezuela, using USDA-ID. Number of colonies (% within location and source).

Location	Morphometric class			
	European	European/Africanized	Africanized/European	Africanized
Louisiana	150 (100%)			
Monagas:				
unmanaged	71 (88%)	1 (1%)	5 (6%)	4 (5%)
swarms	29 (47%)	6 (10%)	5 (8%)	22 (35%)
Total	100 (70%)	7 (5%)	10 (7%)	26 (18%)

removed from the body using forceps, immersed in 1 ml pesticide-grade methylene chloride with sodium sulphate as a drying agent, and sealed in a crimp-top vial. One microlitre aliquots of solvent from each vial were analysed by gas chromatography, and the quantities of the alarm pheromone components calculated by electronic integration.

Colony means were used to calculate the Pearson correlation coefficients (r) between the 25 morphometric measures and the 19 defence characters (12 alarm pheromone components and seven defensive behaviour measures) (SAS Institute, 1985). For each group of traits, the colony means were based on the following: morphometric measurements — 10 bees per colony; defence behaviour — 2 replicates per colony; pheromones — 3 samples of 10 stings per colony. The chi-square test of homogeneity (Freund *et al.*, 1960) compared relative frequencies of colonies in the four classes from USDA-ID for unmanaged colonies *vs.* colonies from caught swarms.

RESULTS

As expected, all of the 150 colonies from Louisiana were identified as European. The colonies from Monagas had representatives for all four of the possible classifications from USDA-ID: European, European with African genes, Africanized with European genes, and Africanized. There are interesting differences in the relative frequencies of the four classes if these colonies are also grouped by source, i.e. unmanaged *vs.* swarms (table 1). These data show that even two years after Africanized bees were found in the area, almost half (47%) of the feral population was still European and only 12% of the unmanaged colonies were showing effects of Africanization, even without requeening. These distributions are significantly different, $\chi^2 = 31.81$, d.f. = 3; $P < 0.01$. Means (± s.e.) of the representative characters for the four classes of bees in Monagas, Venezuela and the European colonies in Louisiana, USA are presented in table 2.

Representative correlation coefficients (r) between the morphometric measures and the behavioural and chemical measures are presented in table 3. While most of the correlations are significant, some of them represent only a weak linear relationship, i.e. $r < 0.25$. Only some of the measures are reported as the calculated values of r were similar for several groupings of characters. Seconds to respond to the suede target (Time to target (s)) had values similar to seconds to respond to the alarm pheromone. Number of bees at the entrance at the end of the test (No. bees at 90 s) reflects the correlations of numbers of bees at 30 and 60 seconds. 'No. of stings' is the total number of stings for two suede targets presented for 30 s. Hexyl acetate and butyl acetate are reported as representative because they are the two alarm pheromone components that show the greatest difference in level of production between Africanized and European honey bees (Collins *et al.*, 1989). Isopentyl acetate is reported because it is not different for the two bee types. Forewing (FWLN) and hindwing (HWLN) lengths represent wing measurements, femur (FELN) and tibia (TBLN) lengths represent leg measurements, and angles (AN) 32 and 39 are representative of all the angles used in the morphometric identification procedure. WXWDA and the numerator of the cubital index, CUBA, had similar r values to the two other wax mirror measures. Function 1 and Function 2, the two discriminant functions, and the probability of the sample being Africanized, all calculated by USDA-ID, are also reported.

Several measures had no significant correlations. Within the morphometric group, number of hamuli and CUBB, the distal abscissa of vein M and denominator of the cubital index, were not correlated with either defensive behaviour or alarm chemicals. The number of bees at 0 s (prior to testing) was not correlated with any morphological measure. Angles 29, 30 and 38 were not significantly correlated with defensive measures. Within the alarm pheromone array, isopentyl acetate and 2-heptanone were not significantly correlated with morphometrics, nor

TABLE 2. Means (± s.e.) of colony means for representative morphometric, defensive behaviour and alarm pheromone characters of four classes of honey bees in two locations. Afr = Africanized; Afr/E = Africanized with evidence of introgression of European genes; Euro/A = European with evidence of introgression of African genes; Euro = European.

Morphometric measures	Monagas, Venezuela				Louisiana
	Afr (n = 26)	Afr/E (n = 10)	Euro/A (n = 7)	Euro (n = 100)	Euro (n = 150)
FWLN	8.74 ± 0.03	8.90 ± 0.03	8.85 ± 0.06	9.08 ± 0.01	9.15 ± 0.008
HWLN	4.16 ± 0.01	4.24 ± 0.02	4.21 ± 0.03	4.30 ± 0.06	4.29 ± 0.005
TBLN	3.14 ± 0.01	3.19 ± 0.009	3.20 ± 0.03	3.23 ± 0.005	3.23 ± 0.003
FELN	2.52 ± 0.01	2.58 ± 0.01	2.59 ± 0.02	2.63 ± 0.004	2.64 ± 0.003
WXWDA	2.22 ± 0.01	2.28 ± 0.01	2.29 ± 0.01	2.37 ± 0.006	2.42 ± 0.004
CUBA	0.52 ± 0.005	0.52 ± 0.01	0.53 ± 0.01	0.54 ± 0.003	0.55 ± 0.002
Butyl acetate[a]	0.61 ± 0.10	0.36 ± 0.07	0.44 ± 0.08	0.18 ± 0.02	0.01 ± 0.003
Isopentyl acetate[b]	1.83 ± 0.23	2.30 ± 0.25	3.01 ± 0.61	1.96 ± 0.11	1.91 ± 0.10
Hexyl acetate[a]	0.66 ± 0.13	0.34 ± 0.04	0.44 ± 0.09	0.23 ± 0.02	0.04 ± 0.003
Time to target (s)[c]	0.33 ± 0.21	0.35 ± 0.35	0.00 ± 0.0	0.22 ± 0.07	9.66 ± 0.52
No. stings[c]	89.4 ± 8.7	102.0 ± 10.4	70.4 ± 11.4	83.8 ± 3.6	10.4 ± 1.0
No. bees at 90 s[c]	137.2 ± 20.8	240.2 ± 30.5	140.3 ± 18.5	177.6 ± 16.1	84.2 ± 5.6

[a]for Louisiana, n = 66.
[b]for Monagas, n = 26, 10, 6, 97.
[c]for Louisiana, n = 147.

were they different for the two populations of honey bees (Collins *et al.*, 1989).

The pooled observations from the surveys in both countries were used and, therefore, represent a honey bee population with morphometric, behavioural and chemical variation that encompasses known extremes (Daly & Balling, 1978; Buco *et al.*, 1987; Collins *et al.*, 1982; Collins *et al.*, 1989) and a large homogeneous European portion. It is likely that if the Venezuela sample had in fact been a more homogeneous population of Africanized honey bees, the pooled populations would have shown a much higher correlation between morphometrics and colony defence. In the original study comparing defensive behaviour levels of the Louisiana and Monagas populations (Collins *et al.*, 1982), we classified all colonies in Monagas as Africanized, because they were in an area where Africanization was taking place and no efforts had been made to requeen with European stock. The USDA-ID identifications presented here (table 1) show that some of those colonies were still morphologically European or intermediate, even among the feral bee population. Also, those that were classified as European (table 1), thirteen of the 71 unmanaged (18%) and eight of the 29 swarms (28%), have probabilities of being Africanized that are greater than the highest probability of being Africanized (P = 0.034) found in the Louisiana population, i.e. the Monagas colonies show clear evidence of introgression of African genes. These morphometric results are similar to those being found in the south Texas and NE Mexico population currently undergoing Africanization (Rubink *et al.*, 1992) and are indicative of many levels of hybridization.

The critical test of defensive behaviour as a discriminator between the two honey bee types is whether it would be effective in a hybridizing population. Therefore, we also calculated the correlation coefficients (r) for the Venezuela population alone. These values are presented in table 4. For this population, the defensive behaviour correlations of 'Time to target (s)' and 'No. bees at 90 s' are not significantly correlated with the morphometric measures. Number of stings is significantly correlated with some morphometric characters (the value of r is low), but not with the probability of being Africanized, the final identification criterion.

TABLE 3. Correlations of some morphological measures with aspects of colony defence behaviour and alarm pheromone production for honey bee colonies in Louisiana, USA, and Monagas, Venezuela. Func. 1 and Func. 2 are discriminant functions and Prob. A is the probability of a sample being Africanized, as calculated by USDA-ID.

Morphometric measures	Behaviour[a]			Pheromones[b]		
	Time to target (s)	No. stings	No. bees at 90 s	Hexyl acetate	Butyl acetate	Isopentyl acetate
FWLN	0.318**	−0.393**	−0.169**	−0.647**	−0.681**	−0.024 ns
HWLN	0.128**	−0.186**	−0.057 ns	−0.485**	−0.544**	0.018 ns
FELN	0.324**	−0.308**	−0.191**	−0.595**	−0.641**	−0.014 ns
TBLN	0.170**	−0.137*	−0.098 ns	−0.501**	−0.532**	−0.006 ns
AN32	0.388**	−0.408**	−0.233**	−0.554**	−0.569**	0.016 ns
AN39	−0.372**	0.358**	0.189**	0.092 ns	0.036 ns	−0.121 ns
WXWDA	0.396**	−0.480**	−0.223**	−0.697**	−0.714**	−0.062 ns
CUBA	0.166**	−0.248**	−0.059 ns	−0.321**	−0.310**	−0.003 ns
Func. 1	0.452**	−0.588**	−0.247**	−0.619**	−0.608**	−0.175*
Func. 2	−0.053 ns	−0.010 ns	−0.086 ns	0.209**	0.196 ns	0.078 ns
Prob. A	−0.339**	0.460**	0.165**	0.546**	0.524**	0.127 ns

*$P < 0.05$
**$P < 0.01$
ns = not significant
[a]$n = 289$
[b]$n = 208$

DISCUSSION

There were significant phenotypic correlations among measurements of morphology and defensive behaviour, particularly for traits that are significantly different between Africanized and European honey bees. Such traits are generally highly heritable. Oldroyd *et al.* (1991) reported very high heritability (h^2) estimates (most ≥ 1.0) for the body characters used in morphometric identification of Africanized honey bees. Collins *et al.* (1984, 1987) reported intermediate to high values of h^2 (0.31–0.93) for measures of colony defensive behaviour and intermediate to very high values of h^2 (0.48–1.98) for components of honey bee alarm pheromone. The two chemicals having the highest correlations with morphometrics, butyl acetate and hexyl acetate, also had the highest values of h^2 (1.94 and 1.98).

These relationships might logically suggest that defensive behaviour and alarm pheromone production might also be used for identification purposes. Indeed, a step-wise discriminant analysis (SAS Institute, 1985) of the data reported here, assessing the seven defensive behaviour parameters, determined that total number of stings was the most effective defence character to discriminate the two bee types, with seconds to react to target, seconds to react to pheromone, number of bees at 90 s, and number of bees in the pretest picture usefully adding to the discrimination in that order (Brown, 1993). For alarm pheromone components, hexyl acetate and the ratio of octyl acetate to isopentyl alcohol, in respective order, were discriminatory (Brown, 1993).

However, the significant correlations and discriminant analysis were calculated from combined data representing two discrete populations. Our most serious concerns about discrimination of the bee types is for conditions under which the populations are mixing and/or hybridizing. Under these conditions, as represented by just the Venezuela population, the significant correlations between defensive behaviour and morphology were almost entirely lost. Therefore, colony defence behaviour alone is not an appropriate measure for identification or certification.

As an example, if we use only the behavioural defensive character best correlated to the morphometric measures, number of stings, to classify the colonies in the Venezuelan portion of the study, two-thirds are misidentified. If more than 76 stings (based on the population mean ± 2 s.e.) indicates an Africanized colony, 56% of the colonies are classed as Africanized when they are actually European, and 10% are classed as European when they are

TABLE 4. Correlations of some morphological measures with aspects of colony defence behaviour and alarm pheromone production for honey bee colonies representing a hybridizing population in Monagas, Venezuela, only. Func. 1 and Func. 2 are discriminant functions calculated by USDA-ID; Prob. A = probability of sample being Africanized.

Morphometric measures	Behaviour[a]			Pheromones[b]		
	Time to target (s)	No. stings	No. bees at 90 s	Hexyl acetate	Butyl acetate	Isopentyl acetate
FWLN	–0.013 ns	–0.166*	0.014 ns	–0.551**	–0.577**	–0.056 ns
HWLN	0.071 ns	–0.164*	0.007 ns	–0.500**	–0.505**	–0.024 ns
FELN	–0.014 ns	–0.089 ns	0.024 ns	0.527**	–0.580**	–0.065 ns
TBLN	–0.076 ns	–0.040 ns	0.034 ns	–0.501**	–0.528**	–0.023 ns
AN32	0.105 ns	–0.247**	–0.066 ns	–0.462**	–0.455**	–0.031 ns
AN39	–0.024 ns	–0.169*	–0.071 ns	–0.270**	–0.340**	–0.340*
WXWDA	–0.109 ns	–0.144 ns	–0.033 ns	–0.600**	–0.626**	–0.139 ns
CUBA	0.004 ns	–0.102 ns	0.025 ns	–0.219**	–0.192*	–0.043 ns
Func. 1	–0.089 ns	–0.228**	–0.072 ns	–0.551**	–0.534**	–0.125 ns
Func. 2	0.242**	–0.165*	–0.144 ns	0.180*	0.171*	0.072ns
Prob. A	0.054 ns	0.161 ns	0.007 ns	0.460**	0.435**	0.072 ns

*$P < 0.05$.
**$P < 0.01$.
ns = not significant.
[a]$n = 139$.
[b]$n = 142$.

actually Africanized. This includes two colonies with low sting numbers and a probability of being Africanized of 1.000. As we wish to use identification for certification of stock and selection of breeders, using stings as the criterion would allow significant levels of African genes into the breeding population. As these represent a tropically-adapted genome, uncontrolled inclusion in stocks to be used in temperate areas would be unwise.

Production levels for two of the sting-associated alarm pheromones reported, hexyl acetate and butyl acetate, had strong correlations with morphology and probability of Africanization even in the hybridizing population. However, sampling and analysis for these characters are very time consuming, requiring complicated equipment, gas chromatographs, with skilled operators. Therefore these are unacceptable for regulatory activities. Effective use of colony defence as an identification tool would also be hampered by the large amount of environmental variation inherent in the expression of this behaviour and the difficulty in creating a simple test that is replicable with many different operators. However, the amount of stinging could be a preliminary indication that requeening or more rigorous identification should be pursued. Additionally, reliance on USDA-ID or FABIS to certify breeding stock as being clearly European should not preclude additional evaluation of these bees for acceptable levels of defensive behaviour, or other characters, as suggested by Spivak *et al.* (1988).

Indeed, in October 1991, representatives of the beekeeping community met in St. Louis, USA, and agreed on the concept of a national certification programme for breeder and production queen honey bees (American Bee Journal, 1991). Such a programme would allow for free movement of certified colonies throughout the USA regardless of any existing quarantines related to Africanized bees. It would also serve to certify honey bee stocks for interstate sale. The basis of the certification as recommended is the USDA-ID morphometric procedure to be used for queen-grafting stock. At this time Texas has adopted a certification plan incorporating USDA-ID and FABIS (Van Cleave, 1993).

The results of this study have implications for beekeepers and others coping with the spread of the Africanized honey bee. One is that a portion of the resident honey bee colonies do persist as European in both the feral and managed populations for some years, even in the absence of requeening efforts. These colonies may begin to show increasing levels of defensive behaviour as did the Venezuela European honey bees (EHB). The level of

introgression of African genes into many of the Venezuelan European colonies might account in part for their defensive responses being more like that of the Africanized or clearly hybrid colonies. Also, reports from Venezuelan beekeepers about the pre-Africanization feral population were of small, dark, nasty bees they referred to as *Apis mellifera iberica*. However, the colonies used in the survey that were not from caught swarms had been requeened by Italian stock several years earlier.

Another possibility for the greater defensiveness of these EHB is the presence of AHB in the same apiary. All of the Monagas colonies tested during this study were in closely-packed apiaries of mixed type. Africanized bees are notorious for high levels of robbing behaviour. Because of this behaviour, the presence of Africanized colonies in an apiary might increase the level of guarding by European colonies and thereby increase their tendency to be defensive. In addition, the test of defensive behaviour deliberately stimulates the colonies to give a maximum defensive response and results in large numbers of defending bees in the air and high levels of bee-released alarm pheromone in the apiary. These conditions would tend to increase defence responses in neighbouring colonies as they are tested. Our later personal observations, while working with mixed and single-ecotype apiaries in Venezuela, support this hypothesis. European colonies in mixed apiaries often seemed to be more defensive than when isolated from AHB.

Therefore, the possible influence of Africanized colonies on the behaviour of colonies of European honey bees in a mixed apiary, as well as the undesirability of keeping excessively defensive bees, should encourage beekeepers to be vigilant in removing or requeening the Africanized units. It is also important to remove these local sources of undesirable Africanized drones, particularly during times of queen mating. The most important point presented is that defensive behaviour is a poor indicator of Africanization and field observations using this character need to be corroborated by morphometric identification.

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