

Do morphometrics and allozymes reliably distinguish Africanized and European *Apis mellifera* drones in subtropical Mexico?

W DE J MAY-ITZÁ; J J G QUEZADA-EUÁN*; L IUIT; C M ECHAZARRETA

Facultad de Medicina Veterinaria y Zootecnia,
Universidad Autónoma de Yucatan, Apartado Postal
4-116, Merida, Yucatan, Mexico

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SUMMARY

Drones reared in colonies of honey bees (*Apis mellifera*) of European (EHB) and Africanized (AHB) origin were characterized using morphometrics and allozyme analyses. 17 characters of the forewing were compared at the univariate and multivariate level using principal component analysis (PCA). Additionally, Mdh and Hk allozyme frequencies were compared between both drone types. Only 5 forewing characters were statistically different between the two drone types and PCA failed to separate clearly AHB from EHB drones. The Hk allele 1 was more frequent in EHB drones compared with AHB ($P < 0.01$). However, the frequencies of the Mdh1 allele in EHB drones from Yucatan was intermediate between AHB and EHB drones from an Africanized-free zone ($P < 0.01$). These results suggest that, for Yucatecan populations, Hk is more informative concerning the African or European origin of drones than Mdh. Evidence of undetected levels of africanization with morphometrics alone and the non-neutrality and high within-population variation of the Mdh loci make the use of these techniques questionable as a diagnostic of africanization in drones from the Yucatan. The use of Hk in combination with mitochondrial and/or nuclear DNA markers would be of more value to analyse the dynamics of male production, seasonal abundance and male releases in drone congregation areas in Yucatan.

*Corresponding author: qeuan@tunku.uady.mx

INTRODUCTION

Africanized honey bees (AHB; descendants of *Apis mellifera scutellata*) arrived in Yucatan in 1987, in an area with a high density of European honey bees (EHB) and much beekeeping activity (Quezada-Euán *et al.*, 1996). Most studies following the process of africanization in the Yucatan and elsewhere have focused on the morphological and genetic variability of workers of European and African descent. Thus, using worker variability as an indicator of africanization, several studies have revealed that the pace of AHB/EHB admixture in this area has been slow and that the frequency of markers of European origin is still high in managed and feral colonies (Rinderer *et al.*, 1991; Quezada-Euán & Hinsull, 1995; Quezada-Euán & Medina, 1998; Clarke *et al.*, 2001).

Few studies have looked into the variability of drones as an indicator of africanization mainly because their presence in colonies is time restricted. However, allozyme and morphometric surveys conducted in drone congregation areas (DCA) have been proposed as accurate estimates of the levels of africanization in a locality (Eischen & Rubink, 1997). Moreover AHB and EHB drone classification would be useful in following the dynamics and seasonal distribution of both types of males especially in drone congregation areas and

thus better understand the biological basis of the process of africanization (Loper & Fierro, 1991). However, for such a study it will be necessary to establish that morphometrics and allozyme analyses can reliably distinguish between drone types.

In Yucatan, drones are produced in one season from December through May. However, evidence for differences in the timing of drone production in EHB and AHB colonies has been reported by Echazarreta & Paxton (1997), with AHBs producing drones earlier in the spring than EHBs. The total numbers of drones produced across the entire year by each colony type are roughly the same in Yucatan.

In this study, drones reared in colonies of honey bees of EHB and AHB origin were characterized using morphometrics and allozyme analyses.

MATERIALS AND METHODS

The study was conducted at Xmatkuil, Yucatan, Mexico (20° 48' N; 89° 39' W). The climate in this lowland area is subtropical with rain during the summer (c. 1100 mm) and an annual average temperature of 22°C (García, 1973).

Drones were reared in managed colonies whose racial origin was previously confirmed by means of morphometrics and behaviour (Rinderer *et al.*, 1993; Collins & Kubasek, 1982). The experimental colonies included 20 European colonies from Baja California, USA, (EHBBC); 20 European colonies from Yucatan (EHB) and 20 Africanized colonies from Yucatan (AHBY) that were started from feral swarms. Queens from Baja California were brought into the Yucatan with the help of the Ministry of Agriculture (SAGAR). At present, the majority of European queens introduced into Yucatan come from an area that is claimed to be AHB free.

The queens from the experimental colonies were marked and confined within a cage together with a frame containing drone comb to induce oviposition. Two weeks later, the cells containing larvae were sealed, the combs were transferred to an incubator at 35°C to await the emergence of the mature males. Samples of c. 50 drones were collected from each colony and preserved at -86°C until further analysis.

Morphometric analysis

A database was constructed with only drones from colonies AHBY and EHBBC since the latter came from

an AHB-free area. Thirty males from each of these colonies were used in morphometric analyses, i.e. 30 × 20 EHBBC and 30 × 20 AHBY. The right forewing from each individual was dissected and mounted on photographic slides. Seventeen characters of the forewing were measured in accordance with Ruttner (1988). The data were recorded by means of an inverted microscope, a digitizer tablet, a PC and a morphometrics programme (Rinderer *et al.*, 1993).

The data were first compared at the univariate level by means of ANOVA (see Daly & Balling, 1978; Dytham, 1999). Secondly the data were submitted to a multivariate approach using a principal component analysis (PCA). The drone scores of the first six components from PCA were compared by means of ANOVA and plotted against pairs of factors (Wiley, 1981).

Allozyme analysis

Fifteen drones from each colony, 300 from each source, were used in the analyses. Two allozymes whose variants show different proportions between EHB and AHB colonies were used in this part of the study: malate dehydrogenase (Mdh), with alleles 1, 2 and 3 (Nunamaker & Wilson, 1981), and hexokinase (Hk),

TABLE 1. ANOVA of 17 forewing morphometric characters between Yucatecan Africanized honey bee (AHBY) and European honey bee drones from Baja California (EHBBC).

Character	Mean		SD		F	Prob. F
	EHBBC (n = 600)	AHBY (n = 600)	EHBBC	AHBY		
Forewing length	11.798	11.547	0.067	0.209	1.297	0.269
Forewing width	3.890	3.831	0.044	0.064	0.566	0.469
Cubital index a	0.617	0.549	0.014	0.009	15.693	0.001
Cubital index b	0.391	0.364	0.011	0.014	2.232	0.152
Angle 29	27.039	31.140	0.278	0.372	77.838	0.001
Angle 30	110.976	107.013	1.190	0.853	7.318	0.014
Angle 31	103.147	108.215	0.835	0.645	23.041	0.001
Angle 32	19.156	17.461	0.343	0.494	7.937	0.011
Angle 33	94.955	93.519	0.682	0.496	2.895	0.106
Angle 34	56.921	55.823	1.272	0.781	0.540	0.479
Angle 35	17.499	18.101	0.671	0.416	0.581	0.464
Angle 36	58.575	58.165	0.555	0.434	0.337	0.575
Angle 38	88.334	85.808	1.437	1.167	1.860	0.189
Angle 39	34.029	34.990	0.676	0.629	1.082	0.312
Angle 40	73.148	76.911	4.499	0.697	0.683	0.428
Angle 41	86.646	86.477	0.841	1.201	0.013	0.910
Angle 42	38.976	33.970	4.384	0.573	1.282	0.272

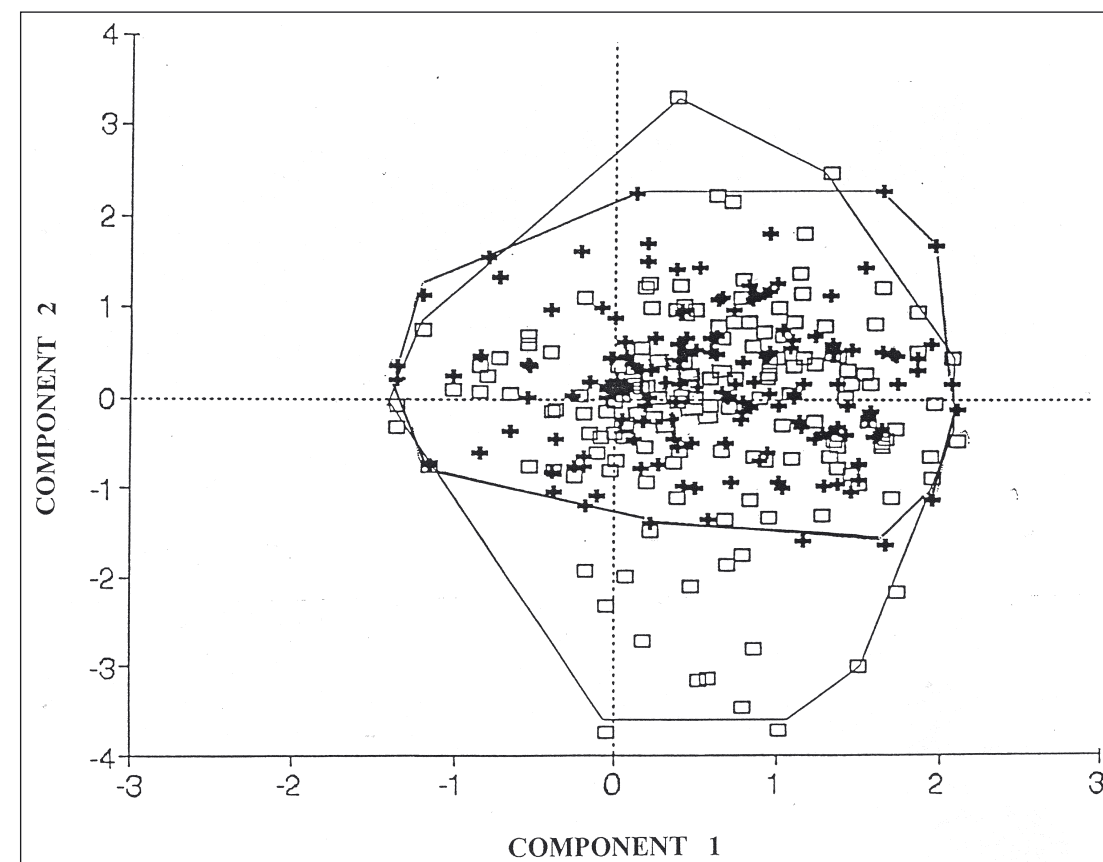


FIG. 1. Distribution of Africanized (+) and European from Baja California (□) drone honey bee scores against principal components 1 and 2 of PCA.

TABLE 2. ANOVA of colony scores after a PCA of European honey bee drones from Baja California (EHBBC) and Africanized honey bee drones from Yucatan (AHBY).

Component	Mean		SD		F	Prob. F
	AHBY (n = 600)	EHBBC (n = 600)	AHBY	EHBBC		
1	-0.553	0.557	0.067	0.068	132.179	0.001**
2	0.152	-0.153	0.063	0.095	7.135	0.001**
3	-0.172	0.178	0.066	0.092	9.495	0.001**
4	-0.007	0.013	0.098	0.060	0.033	0.856
5	0.059	-0.051	0.076	0.086	0.922	0.347
6	0.014	-0.017	0.060	0.099	0.077	0.783

with alleles 1 and 2 (Del Lama *et al.*, 1988). Allozyme variants were detected on polyacrylamide gels (Acrylamide/Bis 37.5:1) using the Bio-Rad IEF mini-cell equipment and the voltage protocols suggested by the manufacturer. The procedure used to extract allozymes in drones was that of Freeman *et al.* (1992) and we followed the staining methods of Hung *et al.* (1991).

Allozyme frequencies were calculated and compared between the drones of the three sources by means of three χ^2 analyses: comparing AHBY vs. EHBBC; AHBY vs. EHBBC and lastly, EHBBC vs. EHBBC (Sokal & Rohlf, 1981).

RESULTS

Morphometrics

At the univariate level only five characters of the forewing showed differences between AHBY and EHBBC drones at the confidence level of 0.05 (table 1). Only one length character was statistically different between both drone types: cubital index a.

The results of the analysis of PCA showed that the drone scores for the first three components were statistically different between AHBY and EHBBC (table 2). However, the plot of drone scores against components 1 and 2 (fig. 1) and against components 1 and 3 (fig. 2) showed no differences in the distribution or separation between both types of drone.

Allozyme analysis

The frequencies of Mdh and Hk variants in drones reared from AHBY, EHBBC and EHBBC colonies are presented in table 3. Although all Mdh and Hk alleles were recorded in drones of all colony types, there were statistical differences in allele frequencies between populations (table 3). The Mdh1 allele was significantly more frequent in AHBY drones (0.833) and less frequent in EHBBC drones (0.173), as expected, since this allele is reported as diagnostic for AHB (Nunamaker & Wilson, 1981). Nevertheless, in contrast, the Mdh1

type was also the most common allele in EHBBC drones (0.493), albeit less frequent than in AHBY drones. The Mdh3 was more frequent in EHBBC drones (0.580) and less frequent in AHBY drones (0.046) with the EHBBC drones intermediate (0.361).

Similarly, the frequencies of Hk1 and Hk2 varied between drone types. Hk1 was significantly more frequent in EHBBC drones and EHBBC (0.97 and 0.91, respectively) compared with AHBY ones. The allele Hk2 was more frequent in AHBY drones (0.980). Both EHBBC populations were statistically different for both Mdh and Hk allelic frequencies. However, the Hk

TABLE 3. Frequencies of isozyme variants in Africanized honey bee drones from Yucatan (AHBY) and European honey bee drones from Yucatan (EHBBC) and Baja California (EHBBC).

n	Drone origin		
	EHBBC 300	AHBY 300	EHBBC 300
Mdh1	148 (0.493)	250 (0.833)	52 (0.173)
Mdh2	44 (0.146)	36 (0.120)	74 (0.246)
Mdh3	108 (0.361)	14 (0.046)	174 (0.580)
Hk1	273 (0.910)	4 (0.013)	292 (0.973)
Hk2	27 (0.090)	296 (0.986)	8 (0.026)

AHBY vs. EHBBC

For Mdh $\chi^2_{(2, d.f.)} = 99.36 < 0.01$
For Hk $\chi^2_{(1, d.f.)} = 485.24 < 0.01$

AHBY vs. EHBBC

For Mdh $\chi^2_{(2, d.f.)} = 279.08 < 0.01$
For Hk $\chi^2_{(1, d.f.)} = 553.02 < 0.01$

EHBBC vs. EHBBC

For Mdh $\chi^2_{(2, d.f.)} = 69.14 < 0.01$
For Hk $\chi^2_{(1, d.f.)} = 11.11 < 0.01$

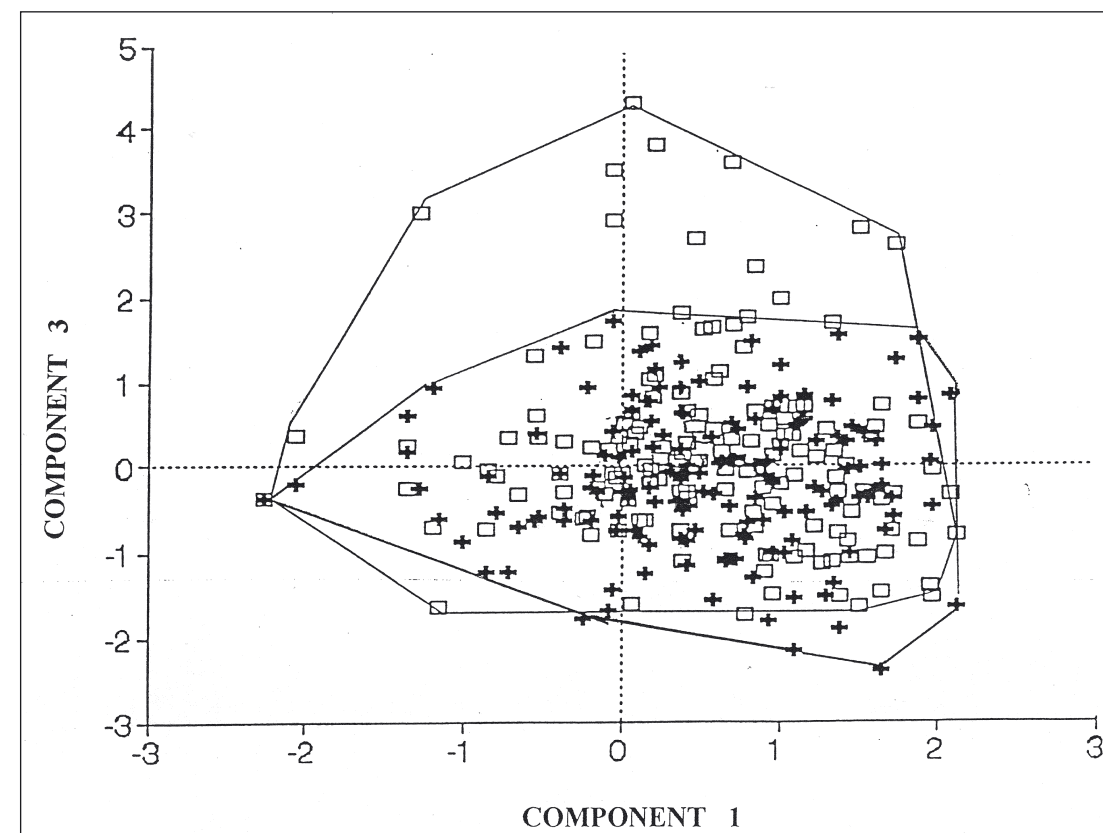


FIG. 2. Distribution of Africanized (+) and European from Baja California (□) drone honey bee scores against principal components 1 and 3 of PCA.

profiles of EHBBC and EHBBC drones were more similar than their Mdh allelic frequencies (table 3).

DISCUSSION

In this study, we found that, for Yucatecan populations, forewing morphometrics and the Mdh1 allele failed to provide conclusive results on the racial origin of drones.

Forewing morphometrics failed to separate drones of EHB and AHB origin. These results are in contrast with the findings of Ruttner (1988) who reported significant variability and separation between drone samples of different racial types analysing 25 characters. One explanation could be that morphometric characters other than those of the forewings may have better discriminant powers in drones. Alternatively, the experimental colonies could have been headed by hybrid queens. However, only extremely defensive and morphometrically defined AHB (probability of africanization ≥ 0.01) colonies were used in this group. Moreover, all AHBY queens originated from feral colonies and there is evidence that feral populations are mainly derived from AHB matriline (Hall & Muralidharan, 1989; Quezada-Euán & Hinsull, 1995). Additionally, dis-

tinctive EHB colonies from Baja California, an AHB-free zone, were used in this part of the study. We think that a more likely explanation is that AHBY and EHBBC males show comparatively less variability in the characters of the forewings compared with workers. For instance, analysing as few as 10 forewing characters in workers, Lobo (1995) was able to separate Costa Rican EHB and AHB. Evidence for low variability in morphometrics of males also comes from the work of Carvalho (1982) who analysed 49 characters but only found differences in seven characters from drone samples of EHB and AHB origin. In comparison, studies where AHB and EHB worker morphometric variability has been analysed using several structures of the body, significant differences have been reported consistently between both racial types and their hybrids (Daly & Balling, 1978; Buco *et al.*, 1987; Rinderer *et al.*, 1990; Oldroyd *et al.*, 1991). It would be interesting to analyse the discriminating power of structures of the body other than the forewings in drones.

The frequency of the Mdh1 allele was found at a low frequency in EHBBC drones. This finding is in agreement with frequencies reported in EHB populations from regions of Mexico other than the Yucatan before africanization and in commercial colonies of European

origin in southern USA (Kitto *et al.*, 1989; Labougle, 1989; Schiff & Sheppard, 1995). However, Mdh1 was found at a high frequency in both EHB and AHBY drones (albeit significantly higher in the latter).

There are several explanations for why the Mdh1 allele was more frequent in EHB from Yucatan compared to those from Baja California. Firstly, africanization could have gone undetected in the parental EHB colonies to an unknown extent because morphometrics alone do not detect intermediate and low levels of hybridization (Guzman-Novoa *et al.*, 1994). Thus, an unusually high frequency of the Mdh1 allele could have been present in this type of colony as a consequence of their hybrid status.

Secondly, undetected levels of africanization could have also concurred with the fact that Mdh alleles are non-neutrally selected in *A. mellifera*. The Mdh locus in this species is under selection due to differential thermostability of its three main alleles (Nielsen *et al.*, 1994; Cornuet *et al.*, 1995; Hatty & Oldroyd, 1999). Since the Mdh 1 and 3 alleles seem more thermostable, they are expected to be at higher frequencies in tropical climates. In this respect both alleles were at high frequency in EHB. Rinderer & Sylvester (1981) proposed that the expression of the Mdh1 allele may be beneficial for bees in tropical areas, and that natural selection may raise the frequency of this allele in such regions. Thus, after 100 years of introduction of European strains of *A. mellifera* into the Yucatan peninsula (Calkins, 1974), it could be that this population has been selected for a higher frequency of Mdh1 because most managed colonies in this area are left to naturally requeen themselves (Rinderer *et al.*, 1991). It is interesting that Kitto *et al.* (1989) and Labougle *et al.* (1989) analysing honey bee colonies prior to africanization found lower frequencies of Mdh1 (0.12 and 0.19) in other regions of Mexico compared to those (0.26) in one locality from Yucatan (Taylor *et al.*, 1991). Additional evidence of a non-neutral Mdh locus is the fact that Hk1 remained constantly high in EHB. Moreover, there was a significant association between the Hk profiles of these colonies with mtDNA of EHB origin (Quezada-Euán & May-Itzá, 2001).

Finally, the use of Mdh as a diagnostic of africanization has also been questioned because of the broad range of variation of the Mdh1 allele, not only between populations but even within EHB colonies (reported frequencies between 13% and 65%) in the same apiary (Kitto *et al.*, 1990). Similarly, this characteristic may explain the fact that EHB drones from Baja California showed different Mdh1 allele frequencies to EHB drones from Yucatan.

All the evidence from the statements above suggests that Mdh allele frequencies are inappropriate to assess the degree of africanization in drones.

In contrast with the high variability of Mdh loci between bee types, the frequency of Hk allele 1 was consistently higher in EHB from Yucatan and Baja California and

lower in AHB started from feral swarms. In studies using bees from Italy, Mexico and Germany analysed for Hk alleles, Del Lama *et al.* (1988) found that the Hk1 allele was fixed in EHBs. Similarly, Hung *et al.* (1991) found that an AHB-specific protein was highly correlated with the absence of the Hk1 allele in samples from USA, South Africa and Mexico. Thus, Hk seems a reliable marker for determining the racial origin of colonies in Yucatan.

Few allozyme systems are available to study population genetics in honey bees (reviewed in Clarke *et al.*, 2001). The results of this study show that only one of the most widely used locus, Hk, shows enough variability to follow patterns of admixture between AHB and EHB from the Yucatan. Although forewing morphometrics and allozyme analyses are powerful and relatively cheap techniques in worker honey bees, their use for the analysis of drone production, seasonal abundance and artificial releases in congregation areas of Yucatan is limited.

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