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THE INFLUENCE OF HYBRIDIZATION BETWEEN AFRICAN AND EUROPEAN HONEYBEES, *APIS MELLIFERA*, ON ASYMMETRIES IN WING SIZE AND SHAPE

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Abstract.—We examined the possible role of hybridization in the invasion process of the African honeybee by testing two hypotheses regarding fluctuating asymmetry (FA), a measure of developmental stability, in wing characteristics: (1) FA should be higher in hybrid versus parental genotypes of African and European races; (2) FA should be lower in African bees compared to hybrid and European workers. Parental and reciprocal hybrid worker genotypes were cross fostered in common-hive rearing environments. We did not find greater FA for wing size and shape in the hybrids compared to both parental types. However, we did find significantly lower FA of shape in the African workers compared to the European and hybrid workers, suggesting that European bees and their hybrids may have compromised fitness relative to African bees. We also found that the two hybrid genotypes significantly differed in overall wing size and shape. If these differences affect wing aerodynamics, then the paternity of hybrids may influence worker performance and could potentially contribute to the loss of European matrilines. Hybridization had few consistent effects on directional asymmetry for wing size and shape. Genotypic factors played a far greater role in determining the effect of hybridization on wing morphology than did differences in rearing environment. Thus, African bees may have lower FA for wing shape (and by inference greater developmental stability) relative to European and hybrid workers, which may contribute to the ability of African bees to displace European honeybee races in invaded regions.

Key words.—Africanized honeybee, *Apis mellifera scutellata*, fluctuating asymmetry, hybrid inferiority, negative heterosis.

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Introduced organisms often have dramatic effects on the biology and genetics of resident species (Pimentel et al. 2000; Simberloff 2000; Huber et al. 2001). Invasive organisms can therefore provide excellent systems for examining the factors that influence gene flow and shape the genetic structure of competing populations (Holway and Suarez 1999; Clarke et al. 2002a; Fewell and Bertram 2002). A recent and spectacular example of a biological invasion involves the African honeybee subspecies, *Apis mellifera scutellata*, which was introduced into Brazil in the 1950s to interbreed with previously imported European subspecies to improve honey production in the Neotropics (Winston 1992). Since then, this tropically adapted race of bees has spread throughout South and Central America and is now in the southwestern United States (Loper et al. 1999; Fewell and Bertram 2002). From the time of its introduction, it was assumed that African and European honeybee races would interbreed, giving rise to the ''Africanized bee'' of Latin America. However, although substantial hybridization occurs when African bees invade areas with resident European populations (Rinderer et al. 1991; Sheppard et al. 1991; Quezada-Euán 2000; Clarke et al. 2002a), over time European characteristics largely disappear (Suazo et al. 1998; Hall 1999; Quezada-Euán and Paxton 1999; Clarke et al. 2001). Thus, gene flow between the races is asymmetrical and results in the displacement of European traits (Hall 1999; Nielsen et al. 1999; Hall and McMichael 2001), although the extent and rate of displacement may be influenced by the size of the preexisting European population (Rinderer et al. 1991; Quezada-Euán and Medina 1998; Clarke et al. 2002b).

A variety of interacting factors may contribute to the loss of European alleles in colonized areas (Page 1989; DeGrandi-Hoffman et al. 1998a, 2002; Hall 1999; Schneider and DeGrandi-Hoffman 2002, 2003; Schneider et al. 2002). African bees may be competitively superior, especially in tropical and subtropical habitats (Hall 1999). Additionally, several lines of evidence suggest that hybrid workers may have reduced fitness. Negative heterosis has often been suggested to explain the repeated observations that hybrid colonies tend to disappear over time unless managed by humans (Spivak 1992; Hall 1999; Hall and McMichael 2001). Harrison and Hall (1993) reported that European-African hybrid workers have lower mass-specific metabolic capacities than either European or African bees, which might negatively affect flight performance and colony dispersal ability. Similarly, Schneider and Hall (1997) suggested that hybrid workers may be less efficient foragers compared to African bees. Reduced metabolism, dispersal ability, or efficiency in workers could result in diminished survival for hybrid colonies, and may help account for the virtual absence of European matrilines in the invading front of African bees (Hall and Muralidharan 1989; Smith et al. 1989; Hall 1999) and the loss of European characteristics in colonized areas (Rubink et al. 1996; Suazo et al. 1998). However, the influence of hybridization and the importance of hybrid inferiority in the spread of the African bee remain controversial (Rinderer et al. 1991; Lobo 1995; Clarke et al. 2002b; Sheppard 2002).

We examined the roles of hybridization and negative heterosis in the African bee invasion process by investigating two types of asymmetry in worker wing characteristics: fluctuating asymmetry (FA) and directional asymmetry (DA). Fluctuating Asymmetry is defined as the variation in small,

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random differences that occur between left and right side structures in bilaterally symmetrical organisms (Palmer 1994). Because the two sides of an organism are produced by the same genome, FA results from the inability of developmental programs to resist environmental perturbations. Thus, FA is often assumed to be negatively correlated with developmental stability and fitness (Palmer 1994; Møller and Swaddle 1997). Several studies have reported increased FA levels in hybrids compared to parentals, perhaps because of the disruption of co-adapted gene complexes and a resultant decrease in developmental stability (Ferguson 1986; Lamb and Avise 1987; Ross and Robertson 1990; Graham 1992). Directional Asymmetry occurs when one side of a bilateral character is consistently larger than the other side (Van Valen 1962). Although DA is less associated with developmental stability (Palmer 1994), it has been found to differ between parents and their hybrid offspring (Auffray et al. 1996; Klingenberg et al. 1998) and transitions between FA and DA have been suggested as indicators of stress in populations (McKenzie and Clarke 1988; Graham et al. 1993; Henshel et al. 1993; Leamy et al. 1999).

Wing asymmetries in honeybees have been examined repeatedly (Brüchner 1976; Clarke et al. 1992; Clarke and Oldroyd 1996). However, to date, only Smith et al. (1997) have investigated the influence of hybridization on FA and DA in wing morphology. These authors examined bees arising from crosses between two European races (*A. m. mellifera* and *A. m. carnica*) and found greater wing venation abnormalities in hybrid workers and increased DA in hybrid drones. The effects of hybridization between European and African bees on wing asymmetries have never been explored. However, because the races belong to different genetic lineages (Sheppard and Smith 2000) and are adapted to different environments (Winston 1992; McNally and Schneider 1992, 1996), hybridization could disrupt co-adapted gene complexes and result in reduced developmental stability.

We examined two hypotheses regarding the manner in which hybridization could affect FA in wing characteristics. First, FA should be greater in hybrid workers compared to both African and European parental genotypes, as has been found in previous studies of hybridization in other species (Graham 1992). Second, given the fact that over time African bees tend to displace both hybrid and European colonies, FA should be lower in African workers compared to hybrid and European genotypes. The first hypothesis provided a test of negative heterosis per se, and the second allowed an assessment of possible greater fitness in African bees in general. We also examined DA in wing characteristics, because hybridization has been found to affect directional asymmetry in bees and other insects (Smith et al. 1997; Klingenberg et al. 1998). Furthermore, any influence of hybridization on wing characteristics could derive from two interacting sources: (1) genetic incompatibilities in developing hybrid brood, and (2) altered brood-care behavior by adult workers in hybrid colonies. We tested for the significance of these factors on wing asymmetries to investigate the influence of hybridization on the invasion of the African bee in the New World, and to determine the relative contributions of genetics and the rearing environment to developmental success in honeybees.

Materials and Methods

Colony Setup and Insemination Design

We examined the effects of hybridization by cross fostering hybrid and parental brood genotypes in common-hive rearing environments. We used instrumental inseminations to create four types of matings: AA (African queens inseminated with the semen from one African drone); EE (European queens inseminated with the semen from one European drone); A-mix (African queens inseminated with equal, mixed volumes of semen from one African and one European drone) and E-mix (European queens inseminated with equal, mixed volumes of semen from the same drone combinations used for the A-mix matings).

All queens and drones used for the inseminations were obtained from three or more unrelated African and European colonies, to ensure genetic variability and prevent inbreeding. The African queens and drones were reared from colonies established from swarms captured in southern Arizona that were identified as African using morphometric and mitochondrial DNA analyses (Crozier et al. 1991; Rinderer et al. 1993). Although the African genotype has been largely retained in the New World (Hall 1999; Loper et al. 1999), there has been some introgression of European alleles during the colonization of the Americas (Sheppard et al. 1991; Sheppard 2002). The European queens and drones used for the inseminations were reared from commercially produced Golden Italian colonies that consisted of a combination of several European subspecies. Thus, our source colonies were not genetically ''pure'' and differed with respect to feral versus managed status. However, these colonies represent the African and European honeybees that exist in the New World and thus accurately reflect the populations that have interacted during the invasion process.

Our insemination protocol resulted in workers that could be visually distinguished based on cuticular coloration. The African queens and drones had black cuticular coloration, and the European queens and drones carried the Cordovan (*cd*) gene for body color. The *cd* trait is a naturally occurring color variant (Tucker 1986) that produces a distinctive light blond color when homozygous, and an intermediate brown coloration when heterozygous. In the colonies arising from African queens, workers of African paternity were solid black or had a black thorax with a distinct black band across each abdominal tergite. In contrast, European-patriline workers in the African-queen colonies had lighter coloration and no banding on the upper one to three abdominal segments. In the colonies arising from European queens, workers of European paternity were homozygous for the *cd* allele and exhibited a uniform blond coloration with indistinct, light-brown abdominal banding. African-paternity workers in the European-queen colonies were darker in coloration and had distinct abdominal banding patterns. The coloration patterns were verified by inseminating African and European queens with only one African or European drone (voucher specimens maintained at the Carl Hayden Bee Research Center). This method of worker identification has been previously used to examine the effects of African versus European paternity on queen development time (DeGrandi-Hoffman et al. 1998a), worker defensive behavior (DeGrandi-Hoffman et al. 1998b),

queen rearing (Schneider and DeGrandi-Hoffman 2002), and the behavior of emerged queens (Schneider and DeGrandi-Hoffman 2003).

The inseminated queens were used as sources of eggs for the different worker genotypes. The colonies that resulted from the inseminations were used as our common-hive rearing environments. Eggs laid by the AA and EE inseminated queens resulted in African and European workers, respectively. Eggs laid by the A-mix queens produced African and African-European hybrid brood (henceforth designated AE). Those laid by the E-mix queens produced European and European-African hybrid workers (designated EA). For workers arising from the mixed-inseminated queens, we examined asymmetries for only the hybrid workers (AE and EA); asymmetries for parental-genotype workers were examined using the progeny of the AA and EE queens. We used the mixed-insemination protocol to obtain AE and EA hybrids for two reasons. First, when African bees first invade an area containing a resident European population, virgin queens will mate with a combination of African and European drones. Mixed inseminations therefore mimic the dynamics of mating that characterize the period when African bees begin to displace European colonies. Second, because mixed matings are characteristic of recently invaded areas, the resulting mixed colonies represent the normal hive environment in which hybrid brood will be reared.

Cross-Fostering Design

The inseminated queens were established in five-frame nucleus hives and expanded to 45-L hive boxes as colony growth warranted. Once all workers in the colonies were progeny of the inseminated queens, we initiated the cross-fostering experiments. Our basic procedure was to introduce four frames of newly laid eggs (one each of eggs from an AA, EE, A-mix, and E-mix queen) simultaneously into one of each type of common-hive environment. To obtain the different frames of eggs, we randomly designated one AA, EE, A-mix, and E-mix colony as a ''brood-source colony.'' We confined each brood-source queen to the center of a prelabeled frame of empty comb that had been conditioned by workers for 24 h to prepare the cells for oviposition. A queen was confined using a 15 × 20 cm wire mesh push-in cage fitted with a 10 × 12-cm section of queen excluder, through which workers, but not the queen, could pass. The cage therefore allowed for normal worker-queen interactions during the confinement period. Each frame with a confined queen was then returned to its brood-source colony. At the same time, we introduced into each brood-source colony a second frame of empty comb to be conditioned by workers.

After 24 h, the confined queen had laid an egg in each cell contained within the caged area. We then removed the frame containing the caged queen, brushed off all workers, removed the cage and the queen, and marked off the edges of the area containing eggs using color-coded pushpins. The queen was re-caged on the second frame of empty comb and returned to the brood-source colony, along with a third frame of empty comb to be conditioned by workers. This procedure was repeated on four consecutive days, resulting in four frames that contained equal areas of eggs newly laid by the same inseminated queen.

On a given day in which frames of eggs were collected, one of each type was introduced simultaneously into a randomly selected AA, EE, A-mix, or E-mix common-hive environment. The introduced frames were placed in the center of each rearing colony, and to the extent possible, were alternated with the colony's existing brood frames. In this manner, over a period of four consecutive days, we introduced into each of four common-hive environments frames of eggs newly laid by AA, EE, A-mix, and E-mix queens.

Seventeen days after the brood frames had been introduced into a common-hive environment, each was removed, all workers were brushed off, and the capped brood within the area delineated by the pushpins was covered with a wire-mesh push-in cage. The frame was then returned to the common-hive colony. The push-in cages used to cover the areas of capped brood were made of wire mesh that contained openings too small for workers to pass through. Thus, the only workers that could appear under the cages were those that emerged from the confined brood. For the following two to four days, each brood frame was checked daily. If emerged workers were present under the cages, all workers outside the cage were brushed off the frame. The caged workers were then quickly brushed into a prelabeled zip-lock plastic bag and immediately placed on ice. In this manner, we collected 150–200 workers from each frame introduced into each common-hive environment. Because worker development typically requires 19–21 days (Winston 1987), our methodology ensured that all collected workers were progeny of the brood-source colony queens. If any of the introduced eggs were eaten by workers in the common-hive environments and replaced with eggs laid by the resident queen, then these would not have developed to the point of emergence by the time we made our collections. All workers were collected within 24 h of emergence, which minimized potential wing damage that may have resulted from contact with the wire cages. All collected workers were killed by chilling and stored in −80°C freezers.

Two trials of the experiment were conducted. The colonies designated as brood-source colonies in trial 1 were used as common-hive environments in trial 2. Likewise, the colonies designated as common-hive environments in trial 1 served as brood-source colonies in trial 2.

Wing Measurements

We randomly selected 35 individuals from each group of AA and EE workers, 35 AE individuals from each group of A-mix workers, and 35 EA individuals from each E-mix group. The genotypes of all workers were identified using the cuticular color markings described above. For each worker, the forewings were removed and each was placed under a glass cover slip on an unlined, white index card. The edges of the cover slip were then mounted to the card using clear cellophane tape. Care was taken to ensure that the tape did not cover any part of the wings. Each wing was labeled as left or right. We also recorded on each index card the worker's genotype, the type of common-hive environment it had been reared in, and whether it was collected during trial 1 or 2 of

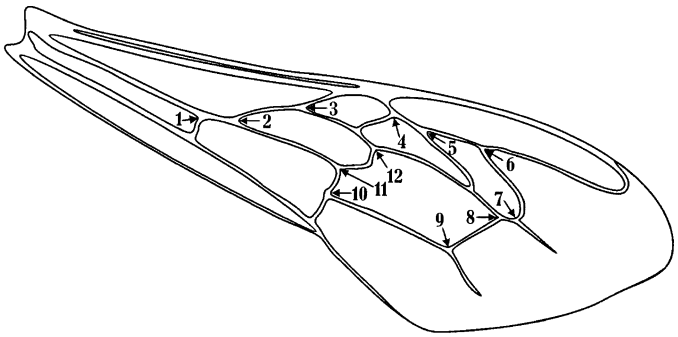

FIG. 1. Landmarks digitized on the forewing of the honeybees.

the cross-fostering experiments. Pooling both trials, sample sizes for each of the 16 combinations of brood and hive genotype varied from 56 to 72 (see Table 2 below); altogether, a total of 1072 bees was available for the analysis.

The left and right forewings of each bee were placed under a camera that projected their image onto a computer monitor. Twelve points located at wing vein intersections were chosen for digitizing (see Fig. 1) that proved reasonably repeatable and that represented the major features of the forewing. These points were recorded in millimeters in *x,y* space using the Measurement TV program (Data Crunch, San Clemente, CA). Only one set of measurements was made on all bees, but two measurements were made on a sample of 24 bees randomly selected from brood and hive genotypes that were subjected to an ANOVA (see below) to assess measurement error.

Size and Shape Characters

The 12 coordinate points digitized on each bee were used to create both size and shape characters. A single size measure, centroid size, was calculated for the left and right sides of each forewing by taking the square root of the sum of squared distances between each landmark and the centroid of each forewing (Dryden and Mardia 1998). The centroid of each side is the point whose coordinates are the means of the *x* and *y* coordinates of all 12 landmarks around the forewing.

Shape variables were created using the Procrustes method (Bookstein 1991; Auffray et al. 1996), which has been adapted for bilateral characters and their asymmetries (Klingenberg and McIntyre 1998). This method used the *x, y* coordinates of the forewings and eliminated variation in size, position, and orientation using four sequential steps that reflected the forewing of one side, and then scaled, superimposed, and rotated the forewing coordinates to produce an optimal fit between corresponding points of left and right sides for all individuals (see Klingenberg and McIntyre 1998). This resulted in 24 new shape characters (*x,y* values of 12 points) that were created for both sides of the forewing in each bee. Generation of these shape characters eliminated four degrees of freedom, resulting in $24-4 = 20$ shape space dimensions (see Klingenberg and McIntyre 1998). Appropriate adjustment for this reduction in number of degrees of freedom was made in the multivariate analyses of variance (MANOVAs) described below.

Once centroid size and the shape characters were calculated for both left and right forewings in each bee, we calculated the mean of the two sides as a measure of overall size and shape in the wings. These measures were subjected to the same analyses as their asymmetries (see below) to discover whether they differed between the brood genotype and common-hive environment groups.

Sources of Variation

A mixed-model, two-way analysis of variance (Leamy 1984; Palmer 1994) was used to assess asymmetry in centroid size in the sample of 24 bees that were repeat measured. In this model, ''individuals'' is a random factor that assesses variation among individual bees, ''sides'' is a fixed factor that assesses directional asymmetry or DA (see below), the individuals x sides interaction assesses FA, and the error assesses variation in the replicate measurements (Leamy 1984; Palmer 1994). If mean squares for the interaction are significant, this indicates that the amount of FA is greater than that due solely to measurement error and thus the asymmetry analysis may proceed (Palmer 1994). To assess asymmetry in shape, a modification of this model known as the Procrustes ANOVA (Klingenberg and McIntyre 1998) was used. In the Procrustes ANOVA, the sums of squares were calculated by adding the sums of squares of all 24 shape characters, and degrees of freedom were obtained by multiplying the degrees of freedom for each factor by the total number of shape dimensions, or 20 (Klingenberg and McIntyre 1998).

The mixed model ANOVAs just described also allowed us to estimate the precision of the replicate measurements. For both forewing centroid size and the multivariate shape characters, variance components were calculated for the three random factors: individuals, the sides x individuals interaction, and error. For shape, this was accomplished by summing all 24 individual variance components for each factor and dividing the total by 20. The magnitude of the error variance relative to that of the sum of these three variances, and especially relative to the individuals x sides interaction (FA) variance, provided appropriate measures of measurement error (Palmer 1994; Leamy 1999).

Asymmetry Characters

To obtain measures of DA for both the forewing size and shape measures, right minus left side differences first were calculated for all individuals. If these *signed* differences were significantly different from zero for either centroid size or shape, this suggested that DA was present (Van Valen 1962). Skewness and kurtosis statistics calculated for the signed differences between sides in each of the characters showed that their distribution was normal, and thus that there was no apparent antisymmetry, a third type of asymmetry often detected by significant platykurtosis (Palmer and Strobeck 1992).

To obtain measures of FA for both forewing size and shape, we estimated error variances from two-way ANOVAs (centroid size) and Procrustes ANOVAs (shape) in the manner already described, but for each of the 32 brood-genotype-by-common-hive environment by trials combinations. These error variances contain both measurement error and FA, but

TABLE 1. Analysis of variance of forewing centroid size and shape in 24 bees that were digitized twice. Sums of squares, mean squares, and variance components are in square millimeters ($\times 10^4$) for centroid size and in dimensionless Procrustes units ($\times 10^6$) for shape. The percentage contributions (%) of each variance component to the total variance also are given.

Source	SS	df	MS	Variance component %	
Forewing centroid size					
Sides	998.83	1	998.834**		
Individuals	9415.54	23	409.370**	100.871	97.04
Individuals × sides	135.41	23	5.887**	2.807	2.70
Error	13.15	48	0.273	0.273	0.26
Forewing shape					
Sides	371416.60	20	18570.83**		
Individuals	16788.70	460	36.497**	6.337	51.49
Individuals × sides	5128.15	460	11.148**	5.178	42.07
Error	760.11	960	0.792	0.792	6.44

** $P < 0.01$.

the error in measurement was assumed to be small and these variances seemed particularly appropriate since FA is actually a populational, rather than an individual, measure (Leamy 1984; Palmer 1994).

Tests for Genotype and Hive Differences

Centroid size and the DA and FA measures of centroid size were subjected to a split-plot ANOVA to test for the significance of differences in trials (T), brood genotypes (B), and common-hive environments (H). In this design, T and the T by H interaction were considered random variables, whereas B (3 df) and H (3 df) were considered fixed variables. A major feature of the split-plot design is that two error variances are created, one (with 3 df) used for testing the whole plot factor H, and a second used for testing the subplot factor B and the B by H interaction. If either B or H effects proved significant, three orthogonal, single-degree-of-freedom planned contrasts were conducted: a contrast of parents (AA and EE pooled) versus the hybrids (AE and EA pooled), a contrast of the two parents (AA versus EE), and a contrast of the two hybrids (AE versus EA). These planned comparisons allowed us to examine our first hypothesis (e.g. greater asymmetries in hybrids vs. parentals), as well as investigate possible differences within the hybrid and parental genotypes. In addition, a planned, nonorthogonal contrast of the AA genotype versus all others (EE, AE, EA pooled) was conducted (with an adjusted significance level; Rice 1989) to test our second hypothesis (e.g. lower asymmetries in African bees vs. hybrid and European workers). A restricted maximum-likelihood approach was used for this analysis as implemented in the MIXED procedure of SAS (SAS Institute 1997).

The shape and DA and FA shape measures each were subjected to split-plot MANOVAs of this same design. Due to the four degrees of freedom that were lost during the Procrustes procedure, however, each of these three analyses were run with only 20 of the 24 characters (both values at two landmarks omitted). The 20 characters chosen were arbitrary since identical statistical results are produced in such analyses regardless of which two landmarks are omitted (Klingenberg et al. 2001). The FA shape measures (error variances) were first logged to promote homoscedasticity (Leamy 1984). In these MANOVAs, the degrees of freedom in all cases were 20 times those for the centroid size/asymmetries analysis.

Depiction of Effects on Shape

Although differences in centroid size or its asymmetries among brood genotypes or common-hive environments can easily be depicted by the means of these characters, this is not useful for the multivariate shape characters and shape asymmetries. Instead, differences between the means for all bees versus those for the B or H groups at each landmark were graphed directly onto a diagrammatic representation of the mean forewing. These differences were displayed as lines drawn from the mean location of each point, and represent the magnitude and direction of the change for a particular group (Klingenberg and McIntyre 1998). Because these differences were quite subtle, they were multiplied by a factor of 100 for shape and 1000 for the shape asymmetries in the figures to make them more visible.

We also ran a principal component analysis (PCA) on the shape characters and their asymmetries to describe their covariation in a smaller number of dimensions (Klingenberg et al. 2001). Separate PCAs were run on the covariance, rather than correlation, matrices of three shape characters because these matrices preserve the Procrustes metric and thus do not eliminate this common scale for shape variation (Dryden and Mardia 1998; Klingenberg and McIntyre 1998). Principal Component Analysis reduces the overall variation into a smaller number of variables (PCs) that are useful in describing the variability in shape. We graphically displayed the pattern of landmark displacements of the first two PCs to interpret these PCs as features of shape variation (Klingenberg and McIntyre 1998). We also calculated component scores for the first PC and plotted them against those of the second PC to facilitate inspection of the patterns of these effects throughout the brood-genotype and common-hive environmental groups.

RESULTS

Assessment of Measurement Error

Table 1 presents the results of the analysis of variance of forewing centroid size and shape for the 24 bees that were

TABLE 2. Means ± standard errors for centroid size (CENT), and the DA (C-DA) and FA measures of centroid size (C-FA = error variances × 10^4) of the forewing in bees of each brood genotype that were raised by workers in four common-hive environments. Marginal means over all genotypes also are given. AA, African mother and father; AE, African mother and European father; EA, European mother and African father; EE, European mother and father. *n*, sample size.

Common-hive environment		Brood genotypes				
		AA	EE	AE	EA	Totals
AA	*n*	69	69	72	69	279
	CENT	4.702 ± 0.0078	4.976 ± 0.0100	4.932 ± 0.0068	4.871 ± 0.0083	4.871 ± 0.0075
	C-DA	0.003 ± 0.0029	−0.000 ± 0.0031	0.010 ± 0.0030*	−0.003 ± 0.0031	0.003 ± 0.0015
	C-FA	2.808 ± 0.1364	3.328 ± 0.3618	3.294 ± 0.1206	3.327 ± 0.8419	3.189 ± 0.1953
EE	*n*	69	69	69	70	277
	CENT	4.765 ± 0.0119	4.983 ± 0.0090	5.009 ± 0.0129	4.861 ± 0.0153	4.904 ± 0.0086
	C-DA	0.002 ± 0.0032	0.002 ± 0.0036	0.000 ± 0.0029	0.007 ± 0.0034	0.003 ± 0.0017
	C-FA	3.475 ± 0.1166	4.545 ± 0.4560	2.909 ± 0.4989	4.297 ± 0.4729	3.807 ± 0.2924
A-mix	*n*	56	63	69	61	249
	CENT	4.803 ± 0.0123	4.972 ± 0.0117	4.973 ± 0.0095	4.872 ± 0.0067	4.910 ± 0.0068
	C-DA	0.006 ± 0.0035	0.000 ± 0.0035	0.002 ± 0.0033	0.003 ± 0.0032	0.003 ± 0.0016
	C-FA	3.322 ± 0.1492	3.374 ± 0.9473	3.588 ± 0.0583	3.196 ± 0.6320	3.370 ± 0.2238
E-mix	*n*	63	64	69	71	267
	CENT	4.687 ± 0.0110	4.844 ± 0.0087	4.895 ± 0.0097	4.798 ± 0.0093	4.808 ± 0.0067
	C-DA	−0.001 ± 0.0031	0.000 ± 0.0035	0.006 ± 0.0039	0.005 ± 0.0035	0.002 ± 0.0018
	C-FA	3.546 ± 0.4889	3.995 ± 0.4856	5.272 ± 0.8983	4.376 ± 0.6276	4.297 ± 0.3426
Totals	*n*	257	265	279	271	1072
	CENT	4.738 ± 0.0060	4.945 ± 0.0061	4.952 ± 0.0055	4.850 ± 0.0056	4.872 ± 0.0039
	C-DA	0.002 ± 0.0016	0.000 ± 0.0017	0.004 ± 0.0017**	0.003 ± 0.0017	0.003 ± 0.0009**
	C-FA	3.288 ± 0.1497	3.811 ± 0.2969	3.766 ± 0.3932	3.780 ± 0.3214	3.666 ± 0.1495

* $P < 0.05$; ** $P < 0.01$ in tests of directional asymmetry.

digitized twice. For centroid size, differences between left and right sides are statistically significant, suggesting the presence of DA. Differences among individuals also are significant, and contribute by far the largest amount (97%) to the total variance. The individuals by sides interaction also is significant, suggesting that FA for centroid size is present, although its contribution to the total variance is less than 3%. Measurement error contributes much less than this, however, suggesting it is not an important source of variation in centroid size wing measures. Results for forewing shape (Table 1) also show significance for sides (and thus DA for shape as well), individuals, and the individuals by sides interaction. Variation among individuals (51%) is much less for forewing shape than for centroid size, although the opposite is true for FA, which contributes 42% of the total variation of wing shape. Measurement error variation for wing shape (6%) is greater than that for centroid size, but is still only a fraction of the contribution of FA for shape.

Centroid Size Characters

Table 2 gives the sample sizes, means, and standard errors for forewing centroid size and its asymmetries for bees of each brood genotype raised by workers in each of the four common-hive environments. For centroid size, there is considerable variation in the means among the 16 combinations of brood by worker genotypes, but the marginal means show that there is more variation among brood genotypes than among common-hive environments. In general, centroid size is larger for EE than for AA brood genotypes, and roughly intermediate (between AA and EE) for EA hybrids. Centroid size for the AE hybrids raised in the AA and A-mix colonies tends to be about the same as that for EE bees, but greater than EE bees when raised in the EE and E-mix colonies.

Means for the signed asymmetries of centroid size (Table 2) for each of the 16 genotype-by-hive environments and for the four combined genotypes and hives were tested for significance (difference from zero) by the sequential Bonferroni procedure (Rice 1989). As may be seen, significant DA was detectable only for the AE bees (raised by AA workers, or all workers), and for all 1072 pooled bees. Overall, there seems to be little variation in the signed asymmetries among the various brood genotypes and common-hive environments, although there is some tendency for the AA and especially EE brood genotypes to exhibit less DA of centroid size compared to the hybrids. The centroid size FA measures (error variances × 10^4) also do not exhibit much variation among genotypes, although they are generally lowest for AA bees (mean = 3.29), or for bees reared by AA workers, and higher for the European and hybrid combinations (average = 3.79).

The results of the analysis of variance for centroid size and its asymmetries are given in Table 3. The MIXED procedure does not print out mean squares or the error terms, so only *F*-values and their significance are shown. Differences between the two trials and the interaction of trials with the common-hive environment both generated nonsignificant variances for centroid size and its asymmetries, and are therefore not shown in the table. Centroid size shows significance for brood genotype (B) and brood-genotype-by-common-hive environment (B × H), but not for common-hive environment (H) differences. Judging by the magnitude of the *F*-statistics, differences between brood genotypes are much greater than variation among hive environments within each brood genotype, and in fact show significance even when tested over the B × H interaction (done by pooling over all four hive environments). All four planned comparisons of brood genotypes also show significance, suggesting that forewing cen-

TABLE 3. Results of the analysis of variance, including single degree-of-freedom contrasts, for centroid size (CENT) and the DA and FA measures of centroid size of the forewing in bees. Numerator degrees of freedom (df) as well as *F*-values from the mixed model ANOVA are given.

Source	df	*F*-values		
		CENT	CENT-DA	CENT-FA
Common-hive environment (H)	3	1.70	0.01	3.48
Brood genotype (B)	3	444.18**	1.03	0.91
AA/EE vs. AE/EA	1	164.18**	2.32	0.77
AA vs. EE	1	936.75**	0.41	1.95
AE vs. EA	1	240.08**	0.32	0.01
AA vs. EE/AE/EA	1	1037.85**	0.12	2.72
B × H	9	8.08**	1.43	1.12

** $P < 0.01$.

TABLE 4. Results of the analysis of variance for shape and its asymmetries in the forewing of bees. Numerator degrees of freedom (df) as well as *F*-approximations to Wilks' lambda statistics from the multivariate analyses of variance are given.

Source	df	*F*-values		
		Shape	Shape-DA	Shape-FA
Common-hive environment (H)	60	1.13	0.97	1.07
Brood genotype (B)	60	351.92**	2.32**	1.81**
AA/EE vs. AE/EA	20	168.36**	0.99	1.36
AA vs. EE	20	430.84**	4.69**	2.75**
AE vs. EA	20	453.14**	1.24**	1.32
AA vs. EE/AE/EA	20	346.17**	3.15**	2.14**
B × H	180	4.46**	1.38**	0.81

** $P < 0.01$.

troid size differs between the two parents, the two hybrids, between the pooled parents versus the pooled hybrids, and between AA brood versus all other genotypes pooled. Both DA and FA of centroid size, however, show no significant differences for any of the sources of variation.

In summary, centroid size differed among the four genotypes and was influenced by hybridization. Centroid size was smallest in the AA bees and greatest in the EE workers. Hybridization resulted in a mean centroid size for EA workers that was intermediate between the two parentals, whereas mean centroid size for AE workers was similar to European bees. The four genotypes exhibited few differences in DA or FA of centroid size, suggesting that hybridization had little effect on the asymmetries of overall wing size. Brood genotype had a greater effect on centroid size than did the rearing environment.

Shape Characters

Table 4 shows the results of the Procrustes ANOVA for forewing shape and both shape asymmetries. Trials and the trials by common-hive environment interaction again generated nonsignificant variances for all three shape characters, and are not shown in the table. For shape, the same pattern as for centroid size is apparent: significance for B and B × H, but not for H. Also, the differences between brood genotypes appear to be far more important than the interaction effects, and are significant as well for each of the four contrasts. As was true for centroid size, therefore, parents and their hybrid offspring significantly differ for shape as well.

Signed asymmetries of shape (DA shape measures in Table 4) also show significance for B and B × H effects. The planned comparisons revealed that DA of shape differed between the two parents, between the two hybrids, and between AA versus the others, but did not differ for the pooled-parent versus pooled-hybrid comparison. Fluctuating Asymmetry shape measures differ significantly between brood genotypes, but there is no B × H effect (Table 4). The planned contrasts revealed a significant difference of shape FA between the two parental genotypes but, as was found for DA of shape, FA shape measures did not differ between the pooled-parents versus pooled-hybrid genotypes. In contrast, FA of shape significantly differed between AA workers versus the other genotypes combined (Table 4). The mean error variances (unadjusted for other effects and × 10^6) for the AA, EE, AE, and EA brood genotypes, respectively, are 3.67, 4.40, 4.01, and 4.06, suggesting that the magnitude of the FA shape variance is least in the African bees, most in the European bees, and roughly intermediate between these levels for the AE and EA hybrids. In summary, significant brood genotype differences were seen for shape and both of its asymmetries, and the planned contrasts showed that these differences were primarily between the AA and EE parents, and between AA workers versus the other genotypes combined.

Figure 2 graphically depicts the changes in forewing shape from the overall mean for all 1072 bees for all 4 brood genotypes over each and all common-hive environments. All 16 combinations are shown since there was a significant B × H interaction in the Procrustes ANOVA. This figure clearly shows that the greatest shape differences are between AA and EE brood genotypes, with many of the landmark displacements being in opposite directions for these two genotypes. The shape configurations in both AE and EA hybrids, however, are closer to the overall mean configuration.

Figure 3 shows forewing DA changes in each of the 16 combinations of brood and worker genotypes. DA patterns in these wings are quite erratic among these combinations, although they tend to be less for the four genotypes when pooled over all common-hive environments. Nine of the 12 landmarks in the AA bees tend to show some displacement due to DA whereas this is noticeable for only about four of the points in the EE bees, and even fewer in the hybrids.

Figure 4 shows forewing FA changes for the four brood genotypes only, since H and the B × H interaction were not significant. All four genotypes show noticeable differences from the overall mean in their patterns of displacement, especially at landmark points 6 and 12.

Principal Components Analyses

The first two PCs generated from the principal components analyses previously described accounted for 50% of the variation in shape, 34% of the variation in the signed asymmetry of shape, and 65% of the FA variation of shape. These two PCs are thus useful in describing variation in shape and its asymmetries in only two rather than 20 dimensions, and their patterns of variation for all 1072 bees are shown in Figure

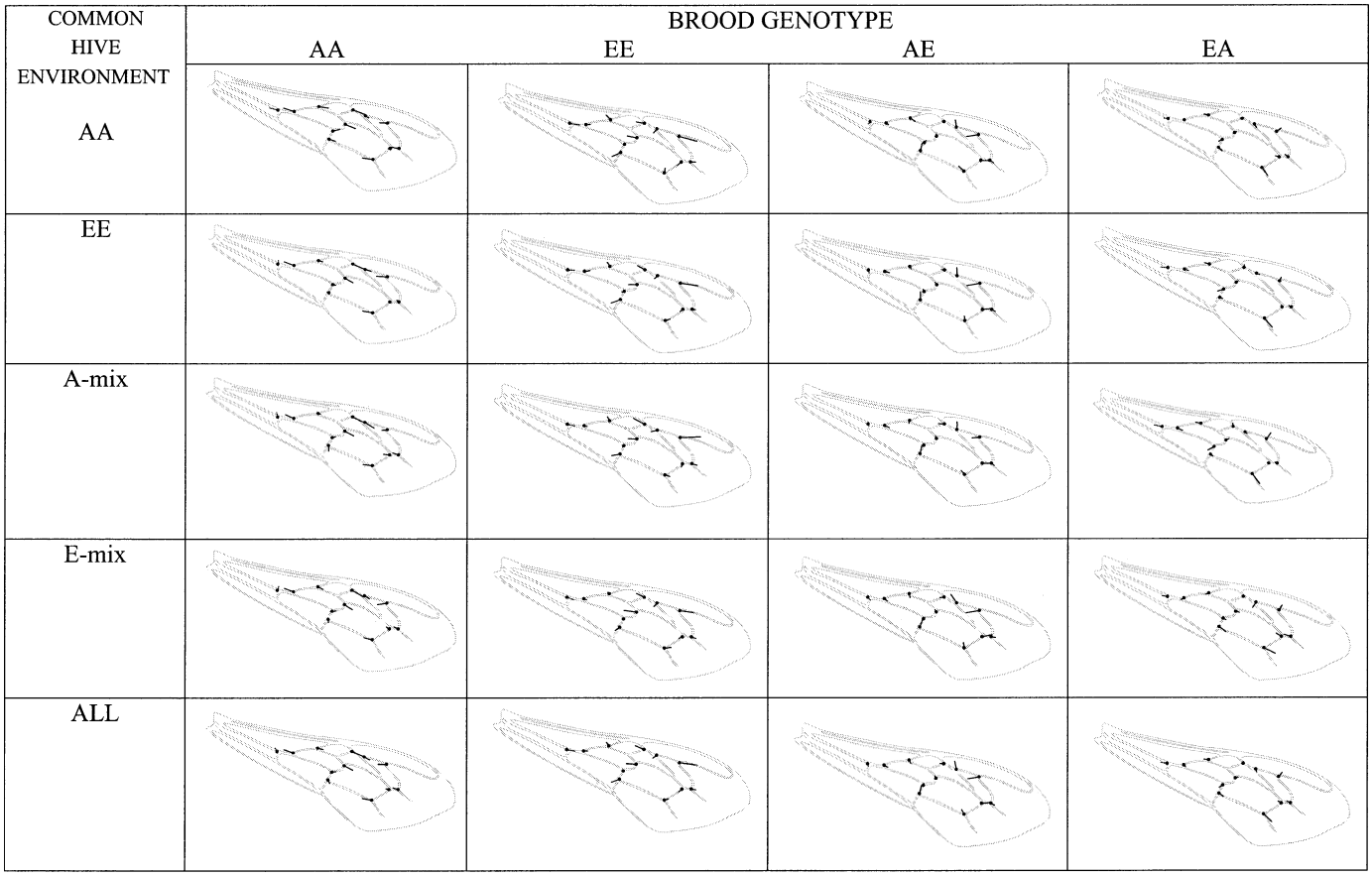

FIG. 2. Forewing shape variation in wings of bees for each combination of brood genotype and common-hive environment. In all cases, landmark shifts are visualized by lines (scaled × 100) that extend from the mean landmark location for all bees (dots) to the mean location for that particular genotype/hive group. The shape changes due to brood-genotype and common-hive combinations are collectively depicted by the direction and length of the landmark shifts (lines). Some landmarks show greater shifts than others, but individual landmarks were not tested for statistical significance since shape is a multivariate trait that is properly tested only for multivariate significance. In general, the AA and EE brood genotypes differ considerably in shape from the overall mean shape configuration for all bees, whereas the AE and EA hybrids are closer to the mean shape configuration.

5. For shape, PCI shows noticeable variation at all but landmark point 4, whereas PCII shows the most shape change in the posterior landmarks. For shape DA, PCI again involves most or all landmark points, but PCII is very different in emphasizing large changes at landmark points 6 and 12. For shape FA, the variation in PCI tends to emphasize displacement at landmark point 12 whereas that for PCII emphasizes displacement at landmark point 6. Most of the rest of the variation is trivial compared to that at these two landmarks.

Results of the plots of the mean PCI and PCII scores for shape and its asymmetries in each of the 16 brood/hive combinations are depicted in Figure 6. For shape, there are distinct clusters of the four brood genotypes, with variation in common-hive environments not being nearly as important. The AA and EE genotypes are separated almost exclusively by PCI, whereas PCII serves mainly to separate the AE and EA hybrids. For the signed asymmetry (DA) of shape, there are not distinct clusters for the brood genotypes, and in fact two of the four AA genotypic groups are widely separated from the other two. For FA of shape, AA and EE brood genotypes are roughly clustered, and except for one EA replicate, both hybrid groups cluster fairly closely together in the two-dimensional space. Basically, therefore, this analysis confirms the greater importance of brood genotype compared with common environmental differences, especially for forewing shape, and the more diffuse pattern of these differences in both shape asymmetries.

DISCUSSION

Fluctuating Asymmetry Patterns

The basic purpose of our study was to test whether hybridization between African and European bees contributed to reduced developmental stability and therefore increased FA in wing characteristics. We investigated two ways in which the effects of hybridization could be expressed: greater FA in hybrids compared to both parental genotypes (an indication of negative heterosis), and lower FA in African workers compared to hybrids and European bees (a possible indication of greater fitness for African bees). These hypotheses seemed reasonable, because it has been suggested repeatedly that European-African hybrids may have reduced viability compared to parentals, and because it is well established that African bees tend to displace hybrid and European

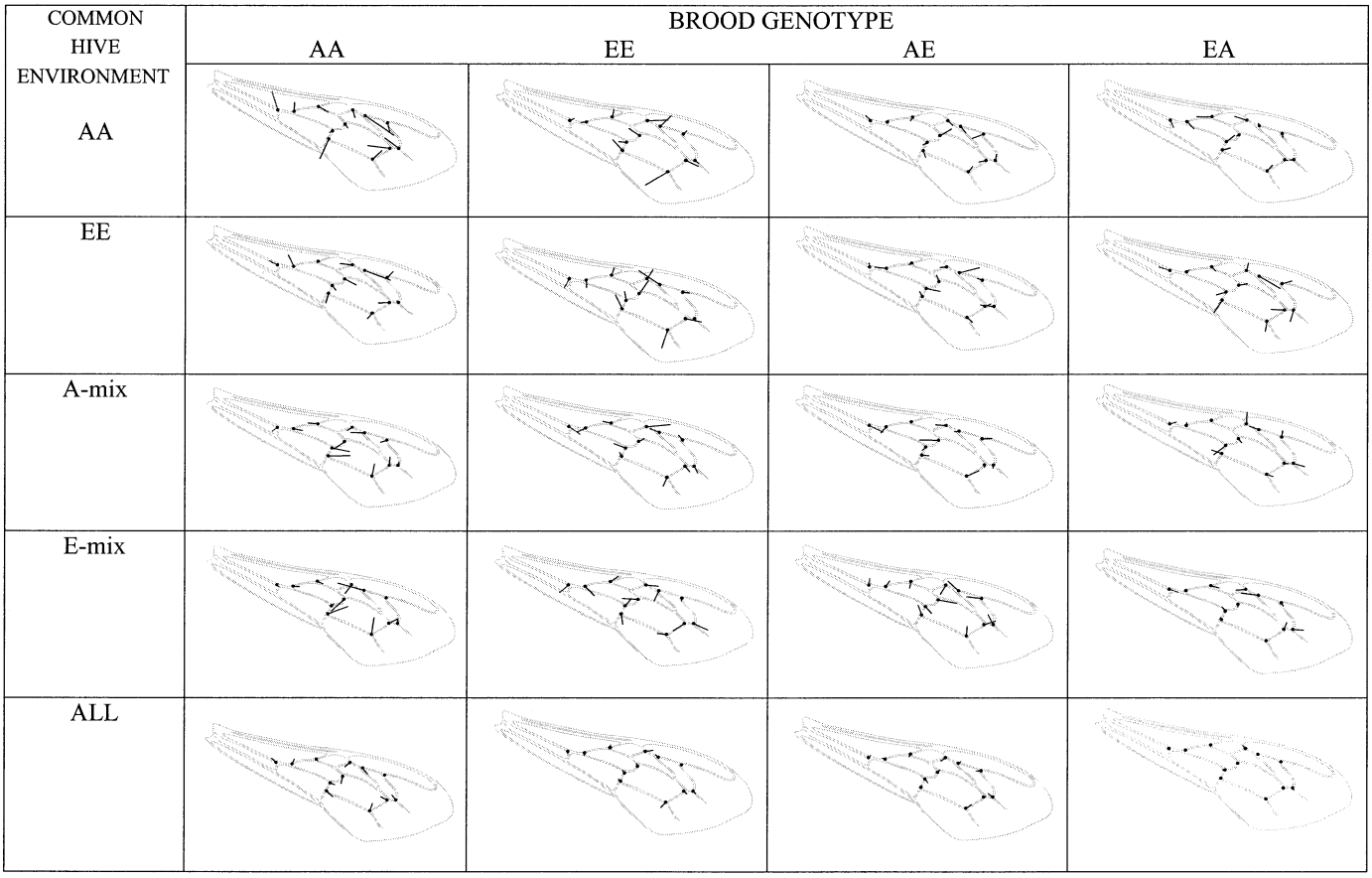

FIG. 3. Forewing variation in signed asymmetry (DA) of shape in wings of bees for each combination of brood genotype and common-hive environment. Landmark shifts are visualized by lines (scaled × 1000) that extend from the mean landmark location for all bees (dots) to the mean location for that particular genotype/hive group. The longest lines indicate the landmarks showing the greatest DA of shape, these being especially noticeable in the AA brood genotype bees.

colonies in invaded areas in the Neotropics (Hall 1990; Spivak 1992; Harrison and Hall 1993; Hall and McMichael 2001; Quezada-Euán 2000). Further, it was assumed that any effect of hybridization on fitness might well be reflected in wing characters, since the overall size and shape of the wing may be critical for normal functioning, including foraging behavior and colony dispersal (Harrison and Hall 1993; Schneider and Hall 1997). Various wing characters have been successfully used to assess FA in bees in previous studies (Brüchner 1976; Clarke et al. 1992; Clarke and Oldroyd 1996; Smith et al. 1997).

With respect to our first hypothesis, we did not find greater FA levels in hybrid workers compared to both parental genotypes for either forewing size or shape, and thus found no direct evidence for negative heterosis per se. Fluctuating Asymmetry in forewing size showed no significant differences among any of the brood-genotype or common-hive environment comparisons. Shape FA did significantly differ among the four brood genotypes, but the hybrid workers were intermediate between the parents, and the AA/EE versus AE/EA contrast failed to reach significance (Table 4). The greatest FA levels for wing shape occurred for EE workers, and this may have been influenced by our use of commercially produced Golden Italian bees that expressed the cordovan color marker. Managed and feral colonies of the same race can differ in wing morphometrics (Rinderer et al. 1993; Quezada-Euán and Medina 1998) and allozyme frequencies (Schiff and Sheppard 1995, 1996), suggesting that management practices may create selective pressures that influence honeybee morphology and physiology. Also, it has been suggested that the cordovan marker may contribute to reduced viability in drones (Tucker 1986; Berg et al. 1997). Because our EE workers were homozygous for the *cd* allele, subviability associated with the cordovan trait could potentially have contributed to reduced developmental stability and greater FA in our European bees. However, there are no known effects of the cordovan marker on the quality and survival of workers and queens (Taber and Wendel 1958; Schneider and DeGrandi-Hoffman 2002, 2003), and the subviability of *cd* drones has been questioned (DeGrandi-Hoffman et al. 2003). It therefore seems unlikely that the increased FA in our European bees was an artifact of the cordovan marker, but rather may have reflected effects arising from human management practices. Mating with feral African bees may have ameliorated these effects to some extent, resulting in intermediate measures for FA of wing shape in the hybrid progeny.

With respect to our second hypothesis, we did find that shape FA was significantly different in the African bees compared to the pooled EE and hybrid genotypes. Most impor-

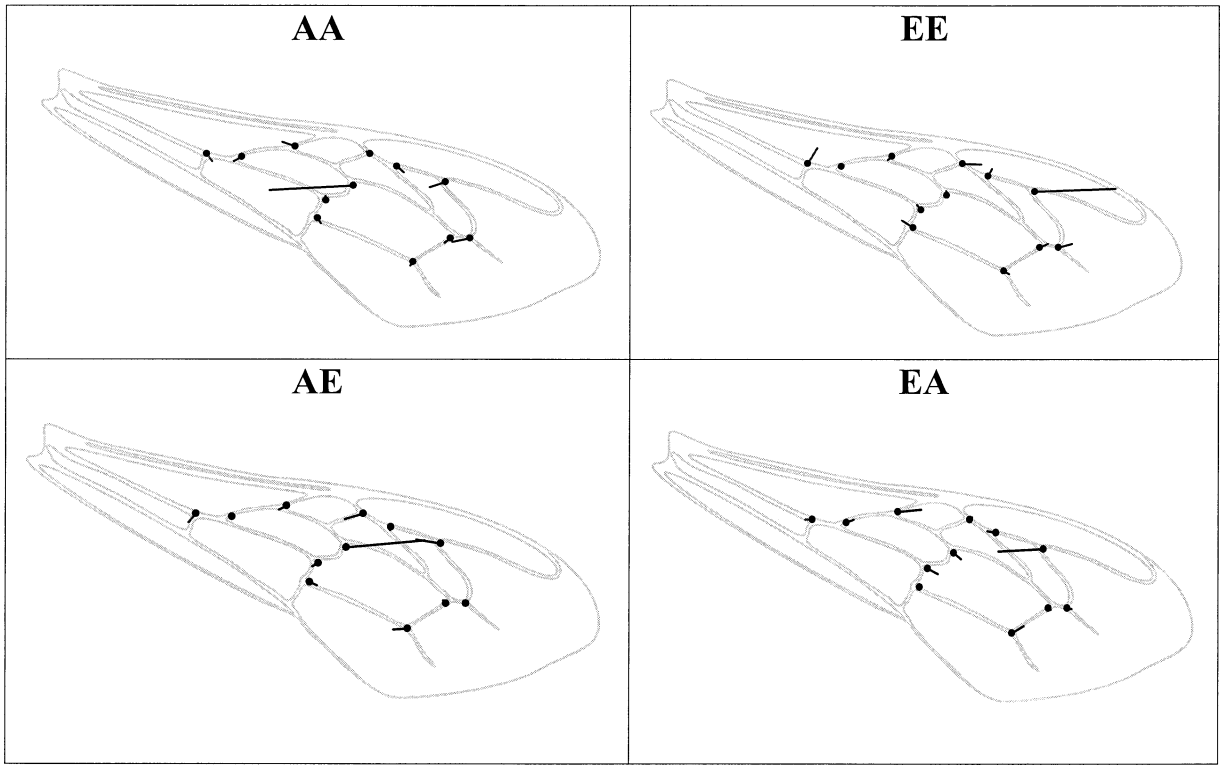

FIG. 4. Forewing variation in unsigned asymmetry (FA) of shape in wings of bees for the four brood genotypes. Landmark shifts are visualized by lines (scaled × 1000) that extend from the mean landmark location for all bees (dots) to the mean location for that particular genotype group. The longest lines indicate the landmarks showing the greatest unsigned asymmetry of shape, and are particularly prominent at landmark points 6 and 12.

tantly, the error variance estimates of the magnitude of shape FA revealed that FA was lowest in the African bees compared to all other worker genotypes. This suggests that developmental stability in fact was highest in our AA bees and lower in the European and hybrid workers. If lower developmental stability and altered wing shape negatively influence worker fitness and performance, then European and hybrid colonies may be less competitive with African bees in invaded areas and may disappear over time. Our results must be interpreted cautiously, because we did not directly examine the long-term survival and success of the hybrid and parental colony types. Nevertheless, the lower shape FA of African bees relative to the other genotypes is consistent with the suggestion that lower fitness may have contributed to the displacement of European traits in areas colonized by African bees in the western hemisphere (Hall and Smith 1991; Hall 1999; Hall and McMichael 2001).

Directional Asymmetry Patterns

Although directional asymmetry is less associated with developmental stability than fluctuating asymmetry (Palmer 1994), we examined DA in our study because it has previously been shown to be influenced by hybridization in honeybee drones (Smith et al. 1997) and in *Drosophila* (Klingenberg et al. 1998). However, we found only subtle and often inconsistent patterns for DA levels for wing size and shape.

For centroid size, significant DA was present in the small sample of 24 bees that were used to estimate measurement error. However, this may have been an unusual sample, since there was little detectable DA throughout most combinations of brood and hive genotypes where sample sizes were larger (Table 2). Nevertheless, DA in centroid size was significant for AE hybrids and approached significance for the EA hybrids ($P = 0.07$), and combining both hybrids produces a mean signed asymmetry of 0.0038 ± 0.0012 that is highly significant ($P < 0.01$). In contrast, the pooled mean for the AA and EE bees (0.0012 ± 0.0012) was not at all close to significance. Although none of the planned comparisons for centroid DA reached significance, our results nevertheless suggest a slight tendency for hybridization to increase DA for overall wing size. Klingenberg et al. (1998) found a similar pattern for forewing centroid size for *Drosophila*, although the difference in DA levels between the two parental lines and their hybrids was significant.

In contrast to the subtle contributions for DA for centroid size, we observed more prominent effects for DA in forewing shape. Shape DA varied significantly among the four brood genotypes, between the two parental genotypes, between the two hybrids and between AA workers versus the other genotypes combined (Table 4). As we found for FA of shape, DA of shape also was lower in the AA workers compared to hybrid and European bees. However, we did not find a significant difference between the pooled parents (AA/EE) versus the pooled hybrids (AE/EA). Similarly, Smith et al. (1997) found that total shape asymmetry (including both DA

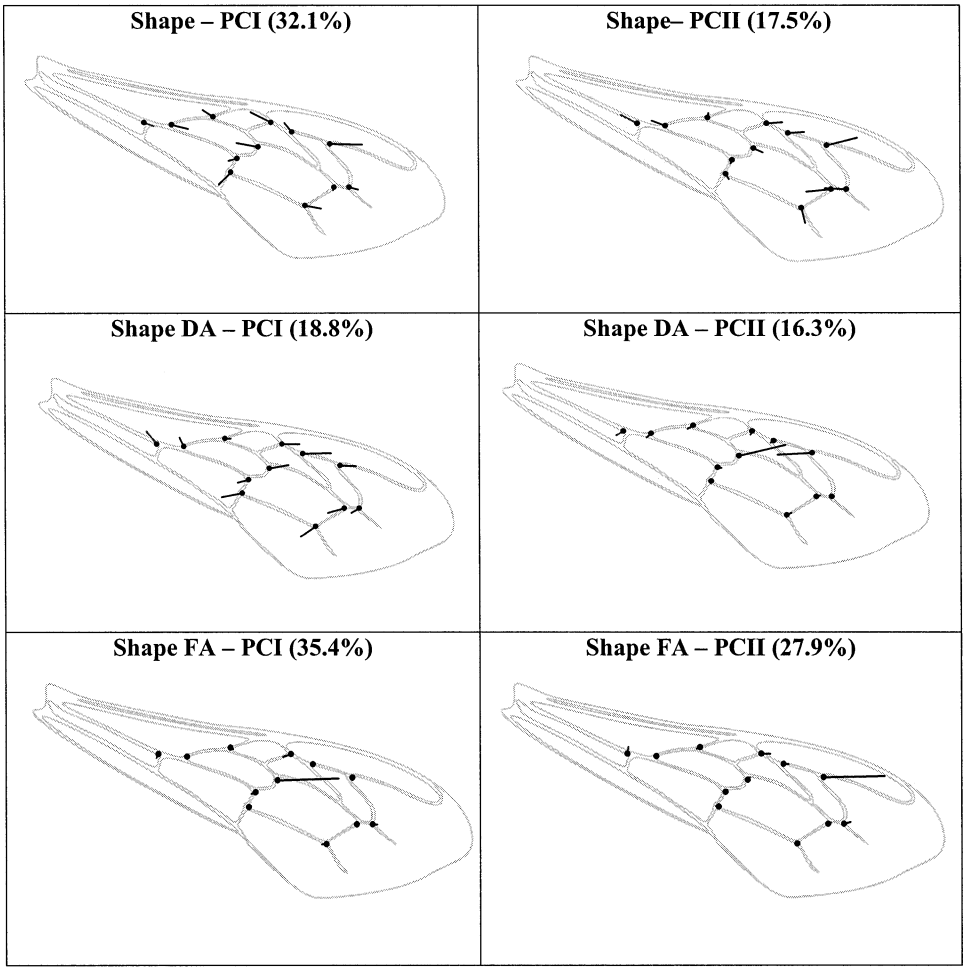

FIG. 5. Principal component analysis of variation in shape and signed and unsigned asymmetry of shape in the forewings of all bees. In each diagram, the pattern of landmark shifts corresponding to the first and second PCs is visualized by lines that extend from the mean landmark location for all bees (dots) to a PC score of 0.12 (shape, shape DA) or 0.02 (shape FA) Procrustes units.

and FA) did not significantly differ between two subspecies of honeybees (*A. m. mellifera* and *A. m. carnica*) and their hybrids, although hybridization did increase DA in the forewing shape of drones.

We did not estimate the contribution of DA to the total variation in shape because differences between sides were viewed as a fixed variable that does not generate a true variance (Leamy 1984). However, if we treat sides as a random variable in a Procrustes shape ANOVA over all trials, broods, and hive genotypes, this analysis yields variance components for individuals, sides, and error, respectively, of 10.24, 0.51, and 4.94. These estimates suggest that FA (plus any measurement error present) contributes 31.5% of the total variation in forewing shape, much more than that of about 3.2% contributed by DA. Thus, in our large sample of bees, FA of shape clearly was far more important than DA of shape. Furthermore, it seems unlikely that the slight effects of hybridization on DA for shape, and the even more minor effects on DA for size, could substantially influence the aerodynamic properties of wings. This, in turn, suggests that changes in directional asymmetry have played little or no role in the invasion process of the African bee.

Size and Shape Patterns

Although patterns of directional and fluctuating asymmetries in the forewings among the worker genotypes were subtle, differences in centroid size and shape were much more obvious (see Fig. 5). Analysis of forewing centroid size showed that African bees were smaller in size than European bees, which is consistent with previous morphometric studies of wing characters (Rinderer et al. 1993; Lobo 1995; Quezada-Euán and Medina 1998). More interesting was the discovery that the two hybrids also significantly differed in wing size. EA hybrids were intermediate between the AA and EE parents, whereas AE hybrids had about the same wing size (mean = 4.952 mm) as their EE parents (mean = 4.945; Table 3). This trend suggests that it may be the paternal parents (drones) that are particularly important in determining overall wing size, since European queens produce smaller-winged progeny if fertilized by African rather than European drones, whereas African queens can produce progeny as large-winged as European strains if they receive alleles from European drones. Paternal effects influenced wing shape as well, since shape also differed significantly between the

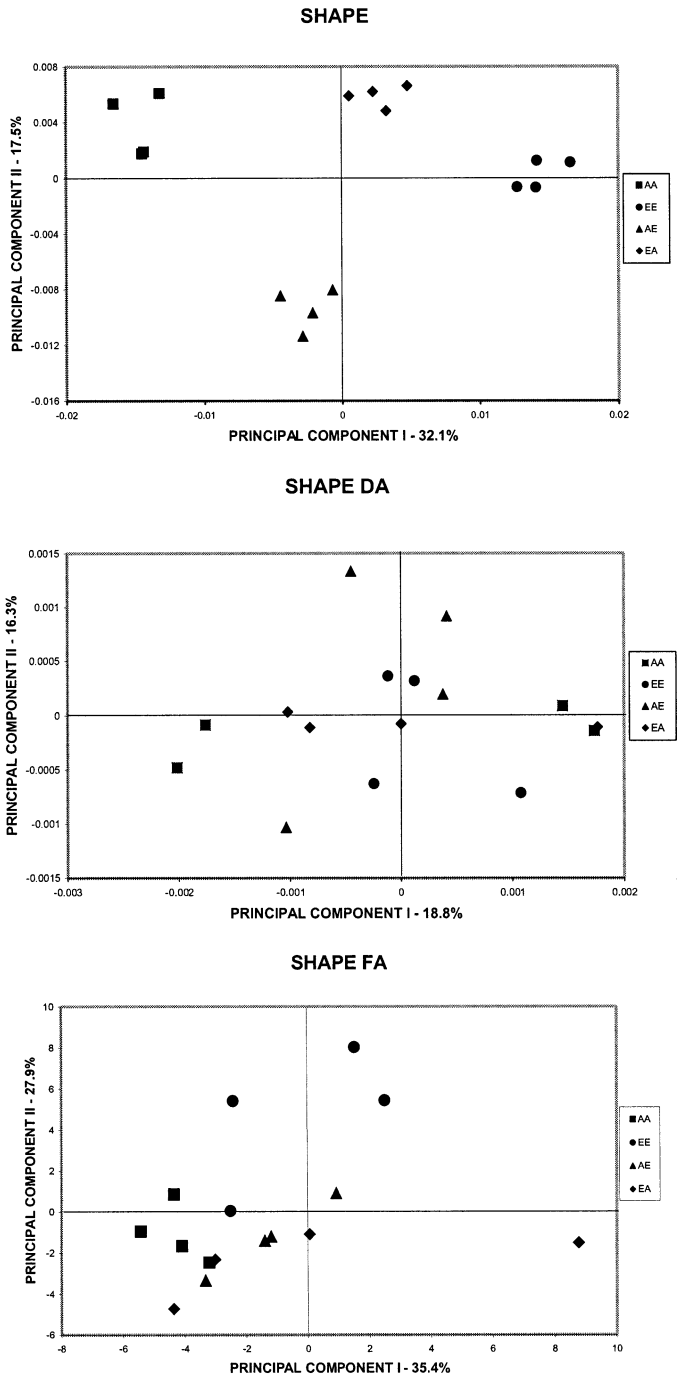

FIG. 6. Scatterplot of the first and second PCs from the principal components analysis of shape and the signed and unsigned asymmetry of shape in the bee forewings. Symbols represent brood genotypes.

two hybrid genotypes (Table 4). These differences in overall wing size and shape could potentially affect flight performance (Hepburn et al. 1999). Similarly, EA hybrids have lower mass specific metabolic rates than AE workers (Harrison and Hall 1993). Taken together, these observations raise the possibility that hybrids with European maternity may experience reduced fitness compared to AE hybrids, perhaps because of genetic incompatibilities between European maternal alleles and African paternal alleles (Hall 1999; Nielsen et al. 1999; Hall and McMichael 2001). Because African colonies often reach high densities in invaded areas (Page 1989; Sousa et al. 2002), queens (both European and African) mate predominantly with African drones. EA hybrids therefore typically far outnumber AE hybrids. If they experience lower fitness, then EA hybrids may play a greater role in determining the dynamics of the African bee invasion compared to AE hybrids.

Common-Hive Environment Effects

Our analyses revealed no effects for common-hive environment on any aspect of centroid size or wing shape. Hive effect was tested over an error mean square that had only 3 df (centroid size) or 60 df (shape), and because this mean square is expected to be larger than that used to test brood genotype, we had much less statistical power to detect significant hive differences. Nevertheless, the marginal means for hive differences for the centroid size characters were very similar (Table 2), suggesting that hive differences would have exhibited no effect even with greater degrees of freedom.

Although we found no common-hive effects, we did find significant brood-genotype-by-common-hive environment (B $\times$ H) interactions for centroid size, wing shape, and signed asymmetry (DA) of shape. Thus, for some of the wing characters examined, the effects of hybridization were expressed differently depending upon a worker's own genotype as well as that of the workers that help to rear it. Genotype-by-hive environment interactions have also been reported for cross-fostering experiments that examined the influence of hybridization on the age at which foraging is initiated (Winston and Katz 1982), the tendency to collect pollen (Guzmán-Novoa and Page 2000) and worker defensive behavior (Guzmán-Novoa and Page 1994; DeGrandi-Hoffman et al. 1998b). Nevertheless, the B $\times$ H effects that we observed were quite small relative to those for brood genotype (Tables 3 and 4). Genotypic factors therefore played a far greater role in determining the effect of hybridization on wing morphology than did differences in rearing environment.

Conclusion

We found lower FA in African honeybees relative to European and hybrid workers, and differences in wing size and shape for EA versus AE hybrids, results that are consistent with previous suggestions that lower fitness relative to African bees and effects of hybridization have contributed to the ability of *A. m. scutellata* to displace European colonies in invaded regions (Hall and Muralidharan 1989; Smith et al. 1989; Hall 1990, 1999; Harrison and Hall 1993; Schneider and Hall 1997). Although the focus of this study was on hybridization and negative heterosis, other investigations have concentrated on a number of other factors that may also contribute to the loss of European honeybee characteristics, such as nest usurpation by African swarms and an African-patriline advantage for reproductives (Vergara et al. 1993; Rinderer et al. 1985; DeGrandi-Hoffman et al. 1998a, 2003; Schneider and DeGrandi-Hoffman 2002, 2003). At present, we do not fully understand the relative importance of these different factors in the spread of the African bee, or how they

may interact with the effects of hybridization to determine the dynamics of the invasion process. Resolving these issues will require long-term studies of the survival and success of AE versus EA hybrid colonies that compete in the same habitats occupied by African bees.

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