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TEMPORAL PATTERN OF AFRICANIZATION IN A FERAL HONEYBEE POPULATION FROM TEXAS INFERRED FROM MITOCHONDRIAL DNA

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Abstract.—The invasion of Africanized honeybees (*Apis mellifera* L.) in the Americas provides a window of opportunity to study the dynamics of secondary contact of subspecies of bees that evolved in allopatry in ecologically distinctive habitats of the Old World. We report here the results of an 11-year mitochondrial DNA survey of a feral honeybee population from southern United States (Texas). The mitochondrial haplotype (mitotype) frequencies changed radically during the 11-year study period. Prior to immigration of Africanized honeybees, the resident population was essentially of eastern and western European maternal ancestry. Three years after detection of the first Africanized swarm there was a mitotype turnover in the population from predominantly eastern European to predominantly *A. m. scutellata* (ancestor of Africanized honeybees). This remarkable change in the mitotype composition coincided with arrival of the parasitic mite *Varroa destructor*, which was likely responsible for severe losses experienced by colonies of European ancestry. From 1997 onward the population stabilized with most colonies of *A. m. scutellata* maternal origin.

Key words.—Africanization, Africanized honeybee, *Apis mellifera*, biological invasion, mitochondrial DNA haplotype, mitotype.

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Upon arrival in a novel environment, a founder population may experience a range of evolutionary trajectories. At one extreme, and most likely, the fate of new introductions is extinction (Williamson 1996). At the other extreme, a founder population may rapidly establish and expand in the new environment (invading population), frequently resulting in severe damage for resident populations, communities, or ecosystems (Pimentel et al. 2000). During establishment and expansion, an invading population may rapidly evolve in response to selection pressures generated by the novel abiotic and biotic conditions (Lee 1999; Huey et al. 2000). At the same time, the invading population may alter the evolutionary pathways of the invaded populations directly by stimulating adaptive responses (Carroll and Dingle 1996) or indirectly by predation (Rodda and Fritts 1997), niche displacement (Race 1982), and competitive exclusion (Holway 1999; Wauters and Gurnell 1999). The impact of competitive exclusion can be further exacerbated by hybridization. If the hybrids formed were sterile, continued immigration of the invading taxon may rapidly drive the resident taxon to extinction (Rhymer and Simberloff 1996; Huxel 1999). Alternatively, if hybrids were fertile and viable, the newly sympatric taxa may merge and the structure of the population would be shaped by immigration and natural selection (Huxel 1999). The evolutionary outcome may range from formation of a hybrid swarm, in which case both taxa would contribute genes to the admixed gene pool (Childs et al. 1996; Perry et al. 2001) to complete genetic assimilation of the resident taxon, in which case its genes would virtually disappear (Levin et al. 1996; Rhymer and Simberloff 1996). Biotic factors (e.g. diseases, parasites) can aggravate the pace and extension of replacement, indirectly by altering the nature of interactions between invading and invaded taxa (McNeil et al. 2003), and directly by causing losses in the resident population (Calvo-Ugarteburu and McQuaid 1998; Rushton et al. 2000).

Studies of the invasive Africanized honeybee (*Apis mellifera* L.) in the United States hold promise for addressing some of these issues because (1) the pre-Africanized managed and feral honeybee populations have been genetically characterized (Schiff et al. 1994; Schiff and Sheppard 1995, 1996; Pinto et al. 2003), (2) Africanized honeybees have recently invaded the United States (Rubink et al. 1996) and formation of a hybrid zone is ongoing, and (3) the arrival of the Africanized honeybee was nearly coincidental with arrival of one of the most destructive pests of honeybees, the parasitic mite *Varroa destructor* (De Guzman et al. 1997).

Africanized honeybees derive from a founder population of a sub-Saharan African subspecies, *A. m. scutellata*, introduced into Brazil from South Africa in 1956 for experimental crossbreeding with temperate-evolved European subspecies in an attempt to create a honeybee better adapted to tropical ecological and climatological conditions (Kerr 1967). Even several hundred years after being introduced in Brazil, the extant European subspecies were poorly adapted and confined primarily to managed apiaries (Michener 1975; Taylor 1988). In 1957, several queens of *A. m. scutellata* escaped from the

quarantine apiary (Nogueira-Neto 1964; Kerr 1967). Since then, the descendents of this tropically adapted subspecies replaced the resident European subspecies and established strong feral populations throughout the American tropics (Taylor 1988; Roubik and Boreham 1990; Winston 1992).

Biological, behavioral, and demographic traits contributed to the rapid replacement of resident European populations by Africanized honeybees. Tropical-evolved honeybees have shorter development period, shorter life span, higher reproductive rates, higher drone production, higher absconding rates, stronger defensive behavior; and forage at younger ages, store smaller amounts of honey, build smaller nests, and are less selective regarding nesting places than temperate-evolved European honeybees (reviewed and extensively referenced in Winston et al. 1983; Winston 1992). These traits conferred high fitness to Africanized colonies allowing them to outcompete managed European colonies and, because European feral colonies were uncommon in the tropics, occupy a virtually empty feral niche (Michener 1975). Additionally, as revealed by morphometrics (Diniz-Filho and Malaspina 1995), allozymes (Del Lama et al. 1988; Lobo et al. 1989; Sheppard et al. 1991a,b; Diniz et al. 2003), restriction fragment-length polymorphisms (Hall and McMichael 2001), random amplified polymorphic DNA (Suazo et al. 1998), and microsatellite markers (Clarke et al. 2002), genetic mechanisms involving hybridization were implicated in the invasion event. Although nuclear markers have shown substantial introgression of European genes, mitochondrial markers have indicated a nearly fixed *A. m. scutellata* mitotype in feral Africanized populations of the neotropics (Hall and Muralidharan 1989; Smith et al. 1989; Hall and Smith 1991; Sheppard et al. 1991b; Diniz et al. 2003). Hypotheses that have been proposed to explain the asymmetrical contribution of nuclear and mitochondrial markers found in Neotropical feral populations include (1) overestimation of *A. m. scutellata* mitotypes (Sheppard et al. 1999), (2) low fitness in F_1 hybrid colonies of European matrilines (Harrison and Hall 1993; Schneider et al. 2003), (3) large differences in population sizes (Page 1989; Rinderer et al. 1991), and (4) reproductive and survival advantages of colonies with queens of *A. m. scutellata* descent (Michener 1975; Rinderer 1988; Taylor 1988).

In spite of their astounding biological success in the neotropics, morphometrical, allozymic, and mitochondrial DNA (mtDNA) data have shown that Africanized honeybees have not expanded into temperate regions of South America, and a complex zone of hybridization has formed in temperate-subtropical boundaries (Lobo et al. 1989; Sheppard 1991a; Diniz-Filho and Malaspina 1995; Diniz et al. 2003; Quezada-Euán et al. 2003). Extrapolating from the climatic limits of Africanized honeybees in South America, Taylor and Spivak (1984) suggested that in the United States, Africanized honeybees would saturate the southernmost regions of some southern states and that a hybrid zone would form northward.

Prior to arrival of Africanized honeybees in 1990 (Rubink et al. 1996), the southern United States supported large well-adapted feral and managed bee populations (Rubink et al. 1990; Kraus and Page 1995; Loper et al. 1999) predominately derived from eastern (*A. m. ligustica*, *A. m. carnica*, and *A. m. caucasia*) and western (*A. m. mellifera*, and *A. m. iberiensis*) European subspecies (Schiff et al. 1994; Schiff and Sheppard 1995, 1996; Loper et al. 1999). The more favorable climatic and ecological conditions for the European honeybees and the large resident European population led to predictions that a greater contribution of European genes to the Africanized gene pool would occur as the Africanization front approached the United States, and that a hybrid zone would form as in southern regions of South America (Sheppard et al. 1991a; Diniz et al. 2003). However, the nearly coincidental arrival in the United States in 1987 (De Guzman et al. 1997) of one of the most serious pests of honeybees worldwide, the parasitic mite *Varroa destructor*, might have compromised in some degree this prediction. Since its introduction, the mite has caused severe losses in managed and feral European colonies (Kraus and Page 1995; Loper et al. 1999). Further, several studies have shown that European colonies are more susceptible to *Varroa* than Africanized colonies (Moretto et al. 1991, 1993; Message and Gonçalves 1995; Guzman-Novoa et al. 1996). Therefore, the combined effect of a depauperate population and differential susceptibility to *Varroa* could accelerate the rate and extension of replacement.

In this study we examine changes across a continuous 11-year period in the mtDNA composition of a feral honeybee population from southern United States (Texas) undergoing both Africanized honeybee and *Varroa* mite invasions. Considering (1) that the population under study lies in the Africanized saturation zone proposed by Taylor and Spivak (1984) where selection may favor *A. m. scutellata* genes, and (2) the differential susceptibility of Africanized and European honeybees to *Varroa* mite, we hypothesize that the invasions should lead to complete replacement of European (non-*A. m. scutellata*) by *A. m. scutellata* matrilines. The study encompasses both the pre- and post-invasion and provides an unusual opportunity to directly observe the interaction of two formerly allopatric populations, at the exact moment of secondary contact, and the role of a third player in the outcome.

Material and Methods

Study Site and Sample Collection

This study was conducted from 1991 to 2001 on the Welder Wildlife Refuge (28°N latitude), located in San Patricio County, approximately 60 km north of Corpus Christi, Texas. The Welder Wildlife Refuge lies in a transitional zone between the South Texas Plains and the Gulf Prairies and Marshes ecoregions (Drawe et al. 1978). Chaparral brushland, scattered mesquite, open grassland, and live oak mottes comprise 80% of the vegetational cover found in the Welder Wildlife Refuge (Blankenship 2000). The climate is humid, subtropical with hot summers and cool winters (Blankenship 2000).

The Welder Wildlife Refuge harbored a large population of feral honeybees with most (85%) colonies occurring in natural cavities of live oak trees (Baum 2003). Cavities occupied by honeybee colonies (active cavities) were located through searches of a 6.25-km^2 study area during periods of high honeybee activity. A limited level of surveys was done initially. As time progressed more exhaustive searches were conducted and greater numbers of active cavities were dis-

TABLE 1. Number of cavities surveyed, new and established colonies from cavities, and colonies from swarm traps. In parenthesis are the number of eastern European, western European, *Apis mellifera lamarckii*, and *A. m. scutellata* scored mitotypes, respectively.

Year	Cavities surveyed[1]	Established colonies from cavities	New colonies from cavities	Colonies from swarm traps
1991	10		10 (7,2,1,0)	
1992	15	9 (6,2,1,0)	4 (2,2,0,0)	15 (3,9,3,0)
1993	34	8 (5,3,0,0)	22 (16,5,0,1)	
1994	70	19 (14,4,0,1)	44 (21,11,9,3)	24 (12,3,4,5)
1995	89	39 (25,4,7,3)	40 (17,6,5,12)	16 (6,3,3,4)
1996	89	19 (9,0,2,8)	13 (4,0,1,8)	6 (2,0,0,4)
1997	89	12 (3,0,1,8)	22 (6,1,2,13)	13 (2,2,0,9)
1998	89	23 (4,0,2,17)	16 (2,1,3,10)	1 (0,0,0,1)
1999	96	30 (4,0,3,23)	31 (3,2,0,26)	
2000	111	51 (4,1,3,43)	27 (5,3,2,17)	5 (1,0,1,3)
2001	112	46 (3,2,3,38)	15 (1,1,2,11)	11 (2,1,1,7)

[1] Some cavities were occupied by more than one colony within a year and some were inactive for variable periods of time. Monitoring of five cavities was discontinued in 1993. Two cavities were in man-made structures (discovered in 1991) and three were in tree cavities (discovered in 1993) located outside of the study area.

covered. Once discovered, a cavity would be examined in all subsequent surveys. A total of 112 tree cavities were included in the study area (Table 1).

Adult honeybee workers (partially sterile females) were sampled yearly from cavities in early (February–May) and late (July–December) seasons, except in 1996 and 1998 when only late season sampling was made. In most years, honeybee workers were also collected from colonies caught in swarm traps (Schmidt and Thoenes 1987; Table 1). Once sampled, swarm trap colonies were sacrificed. Honeybees from sampled cavities and swarm traps were cryogenically preserved on dry ice and transported back to the laboratory where they were stored at −80°C until DNA extraction.

Based on presence/absence of colonies, cavities were scored as active or inactive multiple times per year as follows: two in 1991, two in 1992, three in 1993, six in 1994, six in 1995, two in 1996, three in 1997, one in 1998, three in 1999, two in 2000, and two in 2001. These scores were used to separate colonies into two categories: new and established. The new category included colonies from newly discovered cavities and from recolonization of inactive cavities. The established category included colonies that had survived at least one winter after the cavity was discovered. The 11-year collection comprises 500 honeybees from colonies in cavities and 91 from swarm traps (Table 1). Colonies from swarm traps were included in the new category.

Mitochondrial DNA Analysis

Mitochondrial DNA, which is shared by all individuals in the colony, was extracted from the thorax of a single worker per colony per sampling season using a QIAamp DNA Mini Kit (Qiagen Inc., Valencia, CA). After extraction, template DNA was stored at −20°C until analyzed.

One to three regions of the mitochondrial genome were analyzed through polymerase chain reaction (PCR) in a stepwise fashion (Fig. 1). We used primers that amplify a 485-bp section of the cytochrome *b* gene (Crozier et al. 1991), a 738-bp section of the large ribosomal subunit (ls rRNA) gene (Hall and Smith 1991), and a 1028-bp section of the cytochrome oxidase I (COI) gene (Nielsen et al. 2000). Single PCR amplifications were performed in 5 μl total volume containing 0.5X Taq DNA polymerase buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 2 pM of each primer, 1 μl of template DNA, and 0.25 unit Taq DNA polymerase (Promega, Madison, WI). The PCR temperature profile was 94°C for 3 min followed by 30 cycles of 94°C for 15 sec, 50°C for 15 sec, and 68°C for 5 sec for all three pairs of primers. After the final cycle, an additional 10 min at 72°C was performed. Following PCR, samples amplified with cytochrome *b*, ls rRNA, and COI primers were digested with the restriction enzymes *Bgl*II, *Eco*RI, and *Hinf*I (Promega, Madison, WI) respectively, using the temperature and buffer conditions recommended by the supplier. The total digestion volume was then electrophoresed on a 2% agarose/TBE gel, stained with ethidium bromide, and visualized under UV light.

In animals, mtDNA is typically maternally inherited and does not recombine during sexual reproduction. Therefore, Africanized honeybee colonies maternally descending from *A. m. scutellata* carry *A. m. scutellata*-type mitochondria. The same circumstance applies to eastern European, western European, and *A. m. lamarckii* (non-*A. m. scutellata*) sources. Because this study focuses on the maternal component of the Africanization process, colonies will be referred to as *A. m. scutellata*, eastern European, western European, and *A. m. lamarckii* throughout this article. Colony mitotypes were scored as shown in Figure 1. Colonies determined to carry *A. m. scutellata* mitochondria following *Bgl*II digestion of the

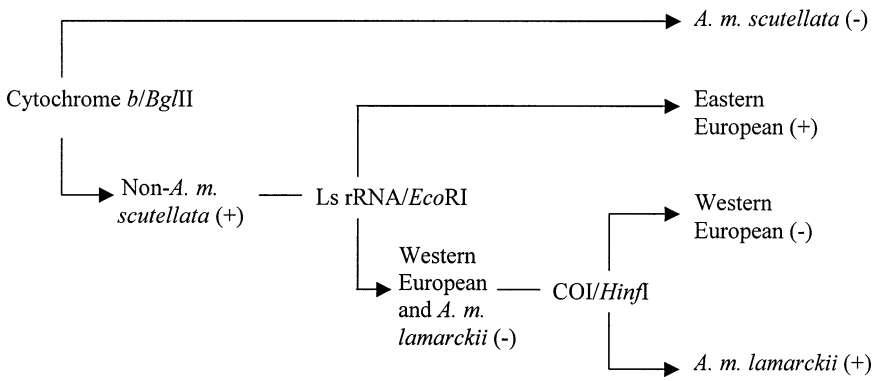

FIG. 1. Flow chart of mitotype determination. (+) indicates presence of a restriction site; (−) indicates its absence.

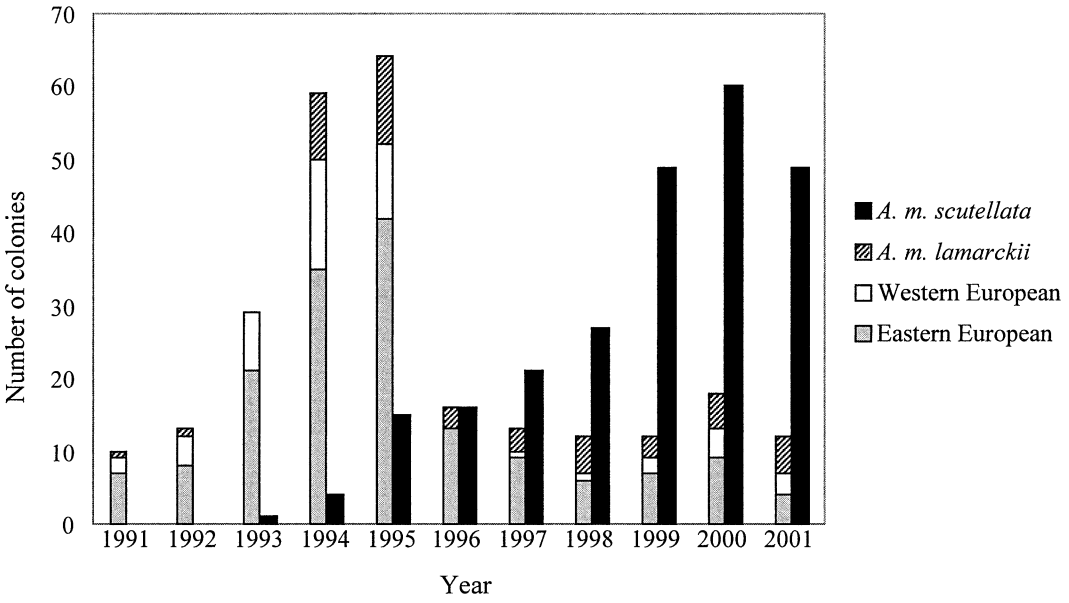

FIG. 2. Temporal mitotype distributions of colonies (new and established) from cavities on the Welder Wildlife Refuge. The stacked column represents non-*A. m. scutellata* mitotypes and the solid column represents *A. m. scutellata* mitotype.

cytochrome *b* PCR-amplified fragment (Crozier et al. 1991; Pinto et al. 2003) were not analyzed further. The Egyptian *A. m. lamarckii* is not discriminated from eastern and western European honeybees using the cytochrome *b*/*Bgl*II assay (Pinto et al. 2003). Colonies that exhibited a non-*A. m. scutellata* mitotype were then PCR-amplified for ls rRNA and digested with *Eco*RI. This assay discriminates colonies of eastern European maternal ancestry from colonies of western European maternal ancestry (Hall and Smith 1991) and *A. m. lamarckii*. Baseline data developed from Old World colonies revealed that *Eco*RI polymorphism does not discriminate western European subspecies from *A. m. lamarckii* (M. A. Pinto, unpubl. data). Thus, colonies exhibiting the lack of an *Eco*RI restriction site were further PCR-amplified for COI and digested with *Hinf*I (Nielsen et al. 2000). *Apis mellifera lamarckii* was identified as having a unique *Hinf*I restriction site within COI (Nielsen et al. 2000).

Statistical Analysis

The homogeneity of mitotype distributions was tested for all pairwise comparisons between years by Fisher's exact test using GENEPOP 3.3 (Raymond and Rousset 1995). Multiple comparisons were corrected for type I error by sequential Bonferroni correction using a global significance level (α) of 0.05 (Rice 1989). The monotonic association between year and mitotype frequency was tested using Spearman's rank order correlation coefficient (r_s) computed using SPSS version 11.0 (Norušis 1993).

RESULTS

Colonies from Cavities

The mtDNA analysis of the 11-year honeybee collection from cavities showed that maternal descendents of eastern European, western European, *A. m. lamarckii*, and *A. m. scutellata* subspecies were each represented in the Welder Wildlife Refuge feral population (Fig. 2). The first colony carrying *A. m. scutellata* mitochondria was detected in 1993. Since then the frequency of *A. m. scutellata* mitotype increased significantly over time ($r_s = 0.98$, $P = 0.000$). Between 1991 and 1993 there were no significant differences in mitotype distributions of the Welder Wildlife Refuge population (Fisher's exact test, $P \geq 0.129$). During this period the maternal composition of the Welder Wildlife Refuge population was 68.0% ± 5.7 (mean ± SD for 1991–1993) eastern European, 26.1% ± 5.5 western European, and 5.9% ± 5.2 *A. m. lamarckii*. However, between 1994 and 1996 the mitotype composition of the population changed radically and the predominantly eastern European population became predominantly *A. m. scutellata* in maternal origin (Fig. 2). Over the 11-year study, the greatest evolutionary change occurred between 1995 and 1996 (Fisher's exact test, $P = 0.000$).

As the Africanization process progressed the mitotype proportions also changed among non-*A. m. scutellata* colonies. There was a marginal decrease ($r_s = -0.69$, $P = 0.018$ is nonsignificant following sequential Bonferroni correction, $\alpha = 0.05$ and $k = 3$) in mitotype frequency of eastern European colonies and a corresponding increase ($r_s = 0.92$, $P = 0.000$) in the mitotype frequency of *A. m. lamarckii* colonies.

The Welder Wildlife Refuge population appeared to have reached stability in maternal composition between 1998 and 2001, because there were no significant changes in mitotype frequency distributions (Fisher's exact test, significant differences at $P < 0.0026$ following sequential Bonferroni correction, $\alpha = 0.05$ and $k = 55$). During this period, 76.7% ± 5.2 (mean ± SD for 1998–2001) of the colonies from cavities exhibited the *A. m. scutellata* mitotype. Among the 23.3% non-*A. m. scutellata*, the mitotype distribution was 47.9% ± 10.5 eastern European, 18.1% ± 7.3 western European, and 34.0% ± 8.9 *A. m. lamarckii*.

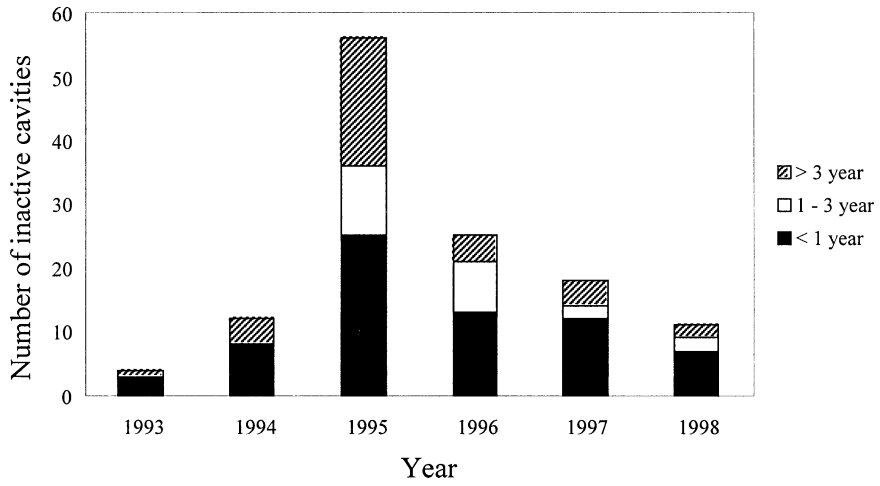

FIG. 3. Recolonization rates of inactive cavities on the Welder Wildlife Refuge. The legend refers to the length of time required before cavities abandoned in a given year were reoccupied. Twelve cavities in 1995, three in 1996, four in 1997, and two in 1998 have never been reoccupied (they were included in the >3 years class).

The largest number of cavities occupied by colonies of non-*A. m. scutellata* maternal descent was observed in 1995. Between 1995 and 1996, most (42 of 79, 53.2%; data not shown) cavities became inactive. During this period, the number of colonies carrying non-*A. m. scutellata* mitochondria dramatically decreased from 64 to 16. In contrast, the number of colonies of *A. m. scutellata* origin increased from 15 to 16 (Fig. 2). Although 53.3% colonies (8 of 15; Table 1) of *A. m. scutellata* mitotype persisted between 1995 and 1996, only 17.2% (11 of 64; Table 1) of non-*A. m. scutellata* colonies persisted. The abrupt decrease in the number of colonies coincided with the arrival of the parasitic mite *Varroa destructor* into the Welder Wildlife Refuge early in 1995 (Rubink et al. 1995). Following Welder Wildlife Refuge population collapse, there was a surplus of vacant cavities that have been reoccupied (Fig. 3) predominantly by colonies of *A. m. scutellata* maternal descent (Fig. 2).

New Colonies from Cavities and Swarm Traps

The mitotype composition of the Welder Wildlife Refuge, shown in Figure 2, was generated from colonies representing overlapping generations, which acts to delay expression of yearly-realized gene flow within a population undergoing a profound evolutionary change. As such, the results for new colonies represent the best estimate of yearly gene flow. Given the surveillance schedule, the number of new colonies from cavities (Table 1) may be underestimated. A colony could die or abscond and have its cavity reoccupied between surveys. Such colonies would be classified as established instead of new. Between 1993 and 2000, 79 of 148 (53.4%) inactive cavities were reoccupied in less than a year. Data obtained for 1993–1998 period suggested a slow reoccupation rate of the remaining vacant cavities (Fig. 3). Seeley (1978) found a lower reoccupation rate in a three-year study of a feral honeybee population of New York State. The relatively rapid rate of cavity reoccupation observed in Welder Wildlife Refuge suggested that recolonizations between surveys could indeed have occurred. An additional source of error could result from colony usurpation. However, a low rate (5–9%) of usurpation of European colonies by Africanized swarms has been reported for managed colonies (Vergara et al. 1989; Danka et al. 1992). If a new colony carried a mitotype different from a replaced colony, then an undetected reoccupation or usurpation would be later identified through mtDNA analysis. Therefore, this error of colony misclassification could be lowered through colony mitotype screening. Mitochondrial DNA analysis revealed 17 undetected recolonizations and/or colony usurpations of 244 (7%) new colonies over the 11-year study, suggesting a low rate of colony misclassification. However, as the frequency of *A. m. scutellata* mitotype increased, the power of identifying colony turnover by mtDNA decreased, due to an increase of the probability of *A. m. scutellata* by *A. m. scutellata* replacements. In this case the frequency of *A. m. scutellata* would be underestimated and the mitotype frequency distribution would be biased. If this were true then the frequency of colonies maternally derived from *A. m. scutellata* that were captured in swarm traps would be consistently higher than in cavities. However, comparisons of mitotype frequencies between swarm traps and cavities showed no significant differences in any year (Fisher's exact test, $0.06 \leq P \leq 1$, Table 1), suggesting that colony misclassification was likely negligible, and mitotype frequencies were not biased. Since mitotype frequency distributions did not differ between new colonies from swarm traps and cavities, we combined both subsamples and estimated mitotype frequencies over time (Fig. 4).

A highly contrasting pattern in mitotype composition was exhibited by the new colonies prior to and after 1996 (Fig. 4). The pre-1996 period was characterized by a temporally heterogeneous mitotype composition with the *A. m. scutellata* mitotype rapidly increasing in frequency since detection of first colony in 1993. During this period, swarms were produced predominantly by colonies of eastern European maternal ancestry, except in 1992 when most (11 of 19, 58%) new colonies exhibited the western European mitotype. This result was unexpected because (1) for the years before 1996 and most years (all except 1998) after 1996 eastern European

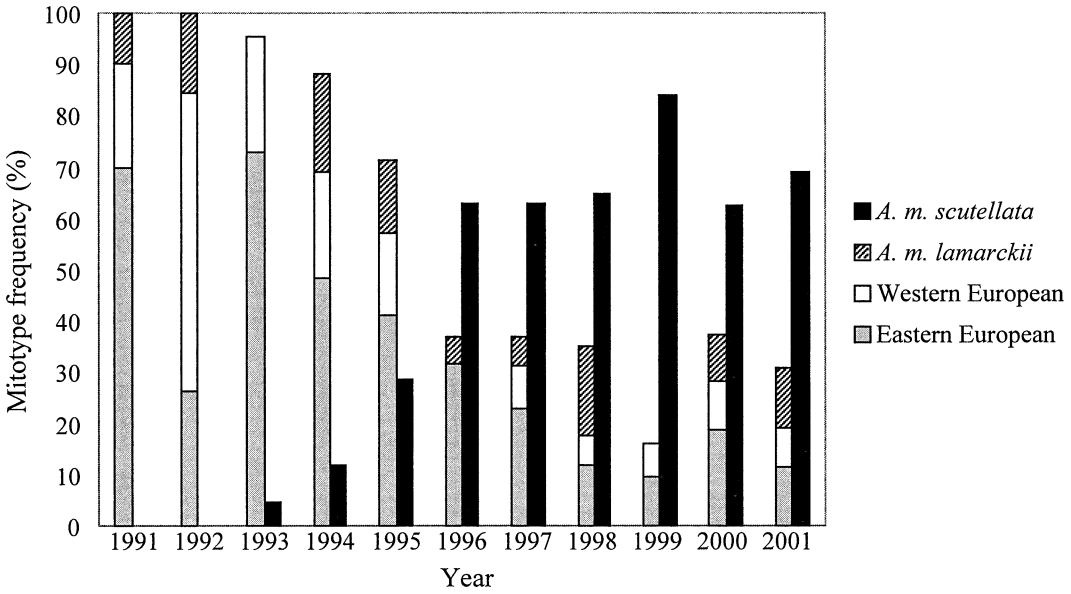

FIG. 4. Temporal mitotype frequency distributions of new colonies (from cavities and swarm traps) on the Welder Wildlife Refuge. The stacked column represents non-*A. m. scutellata* mitotypes and the solid column represents *A. m. scutellata* mitotype.

was the most frequent mitotype among non-*A. m. scutellata* colonies and (2) predominantly eastern European ancestry was found in a large-scale mtDNA survey of feral populations from the southern United States (Schiff and Sheppard 1993; Schiff et al. 1994).

Stability appeared to have been reached during the post-1996 period. Between 1997 and 2001, mitotype frequency distributions were homogeneous (Fisher's exact test, significant differences at $P < 0.0026$ following sequential Bonferroni correction, $\alpha = 0.05$ and $k = 55$), with most swarms (68.6% ± 8.9, mean ± SD for 1997–2001) being produced by colonies maternally derived from *A. m. scutellata*. Among the 31.4% of colonies of non-*A. m. scutellata* maternal ancestry, eastern European mitotype was the most frequent (48.5% ± 12.8), whereas both western European (26.0% ± 8.6) and *A. m. lamarckii* (26.0% ± 19.3) mitotypes exhibited similar frequencies.

The finding that a substantial proportion of non-*A. m. scutellata* mitotypes persisted several years after first arrival of both Africanized honeybees and *Varroa* mites led to rejection of the hypothesis of complete replacement of non-*A. m. scutellata* by *A. m. scutellata* matrilines in the Welder Wildlife Refuge.

DISCUSSION

The 11-year continuous sequence of mtDNA frequencies indicated a dramatic evolutionary change in the Welder Wildlife Refuge feral honeybee population over a very short period of time. Introduction of *A. m. scutellata* mitotypes started at least in 1993. Since then, an increase in the frequency of *A. m. scutellata* mitotypes occurred as more Africanized queens immigrated and/or the recently arrived residents reproduced. The most impressive change on the mitotype composition occurred between 1995 and 1996. In this period, there was a matriline turnover from a predominantly eastern European to a predominantly *A. m. scutellata* population. This evolutionary event was coincidental with arrival of the parasitic mite *Varroa destructor* into the Welder Wildlife Refuge. Although *Varroa* is considered one of the most serious pests of honeybee colonies worldwide, it has been shown that losses are less severe in Africanized than in European colonies (Moretto et al. 1991, 1993; Message and Gonçalves 1995; Guzman-Novoa et al. 1996). Therefore, the finding that between 1995 and 1996 the number of colonies carrying non-*A. m. scutellata* mitochondria dramatically decreased whereas *A. m. scutellata* hardly changed (Fig. 2), suggests that *Varroa* mite played an important role in the change of the mitotype composition of the Welder Wildlife Refuge population.

In addition to colony usurpation and gene flow into the resident European population, Africanized honeybees may expand by winning in the competition for food resources and nest sites (Michener 1975). In the tropics of South and Central America, feral European colonies were uncommon (Taylor 1988; Roubik and Boreham 1990). In contrast, the United States has supported a large well-adapted European feral population (Seeley 1978; Rubink et al. 1990; Kraus and Page 1995; Loper et al. 1999). A density of 11.7 colonies per km^2 was found in the Welder Wildlife Refuge in 1995 (Baum 2003). Only 19% of these colonies were found to be of *A. m. scutellata* maternal descendent. In comparing the Welder Wildlife Refuge results with those from other feral honeybee studies, Baum (2003) suggested that this is the highest estimated colony density for feral honeybees ever reported for a contiguous study area including both suitable and unsuitable habitat. It is thus conceivable that the collapse suffered by the Welder Wildlife Refuge population facilitated the invasion of Africanized honeybees by reducing competition pressure for environmental resources. As colonies succumbed, cavities and food resources would be available to be used by the expanding front of Africanized honeybees and by the swarms produced by surviving colonies. Because Africanized honeybees exhibit a considerably higher reproduc-

tive rate than European honeybees (Winston 1980; Otis 1982), the number of colonies of *A. m. scutellata* mitotype would increase more rapidly than the number of colonies of non-*A. m. scutellata* mitotype. As a result, *A. m. scutellata* colonies would have the potential to occupy the partially empty niche faster than non-*A. m. scutellata* colonies through reproduction and immigration. Therefore, the population collapse combined with high reproductive rate of Africanized colonies and immigration likely resulted in accelerated and increased rates of Africanization in the Welder Wildlife Refuge feral population.

Mitochondrial DNA surveys of honeybees collected in the tropics of South and Central America revealed low levels of European mitotypes (between 0 and 5%) in feral populations (Hall and Muralidharan 1989; Smith et al. 1989; Hall and Smith 1991; Sheppard et al. 1991b; Clarke et al. 2001). On the Pacific coast of southern Mexico, a nearly fixed *A. m. scutellata* mitotype was found in feral populations sampled within a year after first arrival of Africanized honeybees (Hall and Muralidharan 1989; Smith et al. 1989). However, on the Yucatan peninsula, which supported one of the highest densities of managed European colonies in the World prior to Africanization (Quezada-Euán and Hinsull 1995), a greater frequency of colonies of European maternal ancestry was found in feral Africanized honeybees five (20%; Quezada-Euán and Hinsull 1995) and 12 (13%; Clarke et al. 2001) years after migration of the Africanized front. In the Welder Wildlife Refuge, the proportion of non-*A. m. scutellata* mitotypes appears to have stabilized at about 31%, similar to estimates (30%) reported for feral populations from transitional temperate-subtropical regions of Argentina (Sheppard et al. 1991a). This estimate is also similar to the frequency of non-*A. m. scutellata* mitotypes reported for managed (39%) populations from tropical Yucatan (Clarke et al. 2001). The moderate frequency of non-*A. m. scutellata* mitotypes, persisting eight years after detection of first Africanized honeybees in the Welder Wildlife Refuge, is an unusual condition given that the Welder Wildlife Refuge lies in the Africanized saturation zone proposed by Taylor and Spivak (1984) and there was a coincidental severe loss suffered by non-*A. m. scutellata* colonies with expansion of the Africanization front into the Welder Wildlife Refuge. Whether the frequency of non-*A. m. scutellata* matrilines in the post-Africanization period would be greater had the Welder Wildlife Refuge feral population not collapsed is an unanswerable question. Nonetheless, as predicted for the United States, the observed frequency of non-*A. m. scutellata* mitotypes in the Welder Wildlife Refuge population was greater than that reported for feral Neotropical populations. Only future surveys will tell whether the Africanization process is complete. However, the apparent stability reached in the last years of the study suggests that non-*A. m. scutellata* mitotypes are unlikely to be driven to extinction.

The frequency of eastern European, western European, and *A. m. lamarckii* mitotypes found in the pre-Africanization Welder Wildlife Refuge population, was similar to previous estimates from large-scale surveys of the southern U.S. feral population (Schiff et al. 1994). In both studies, colonies of eastern European matrilineal ancestry were the most common and colonies of *A. m. lamarckii* matrilineal ancestry were present in a low frequency. Interestingly, as Africanization progressed in the Welder Wildlife Refuge, a decrease in the relative mitotype frequency of eastern European and an increase in *A. m. lamarckii* were observed. During the post-Africanization period, colonies carrying *A. m. lamarckii* mitochondria became as abundant as those of western European. Further investigation is needed to ascertain whether the observations represent a local effect.

Our results showed that the non-*A. m. scutellata* mitochondrial genomes were not replaced by that of *A. m. scutellata*. Instead, an apparent stable mixture of mitotypes was present in the Welder Wildlife Refuge over the last five years of the study. This finding suggests that the study area lies within what will become the stable hybrid zone.

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