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Forensic Implications of Biochemical Differences Among Geographic Populations of the Black Blow Fly, *Phormia regina* (Meigen)

REFERENCE: Byrne, A. L., Camann, M. A., Cyr, T. L., Catts, E. P., and Espelie, K. E., "**Forensic Implications of Biochemical Differences Among Geographic Populations of the Black Blow Fly, *Phormia regina* (Meigen),**" *Journal of Forensic Sciences*, JFSCA, Vol. 40, No. 3, May 1995, pp. 372–377.

ABSTRACT: Cuticular hydrocarbons were extracted from individual adult blow flies from three geographic populations of *Phormia regina* from areas near Tucannon River and Lyle Grove, Washington, and from Rensselaer, Indiana. The individual extracts were subjected to gas chromatography/mass spectrometry (GC/MS), and 22 hydrocarbons were identified. Discriminant analysis of the cuticular hydrocarbon profiles separated the flies according both to location and gender. These results have potential forensic applications in the determination of corpse relocation and in the study of the population ecology of species and populations.

KEYWORDS: forensic science, black blow fly, corpse relocation, cuticular hydrocarbons, discriminant analysis, forensic entomology, geographic populations, Insecta, Diptera, Calliphoridae, *Phormia regina*

Forensic entomology has been defined as the study of insects as it pertains to legal matters [*1*]. Although insects have been studied and used primarily for estimation of postmortem interval [*2,3*], they have also been used for drug identification [*4*], the determination of antemortem trauma, and to verify corpse relocation [*1,3,5*].

Blow flies are important indicators of corpse relocation in that they are generally the first to discover and oviposit on carcasses outdoors [*2,3*]. Therefore, transportation of the body from the death site frequently results in the transportation of the body's acquired fauna. The geographic distribution of blow fly species varies considerably [*6–12*]. Discrepancies between the composition of insect species on a body and the composition of insect species in the geographic region where the body is discovered provide evidence that the victim was relocated from the site where death occurred [*1*].

The outer surface of all insects is covered with a layer of species-specific cuticular lipids that serve primarily to limit water loss [*13–17*]. Insect cuticular lipids are frequently composed of a complex mixture of hydrocarbons [*13,15,17*]. Lockey [*15*] described the distinct variability in cuticular hydrocarbon profiles among closely related species of insects using gas chromatography/mass spectrometry (GC/MS). Several studies have shown that insect populations of the same species that are located in separate geographic regions may have distinct cuticular hydrocarbon compositions [*18–21*].

Insect cuticular hydrocarbon composition, however, may vary for several reasons. Diet and age of the insect are two variables that must be considered in cuticular hydrocarbon composition analyses. However, Nelson [*22*] and Blomquist and Jackson [*23*] found that only a miniscule amount of hydrocarbons from the diet were incorporated directly into the insect cuticle. This indicates that the majority of hydrocarbons recovered from an insect cuticle are biosynthesized by the insect and that the composition of the cuticular hydrocarbons is, therefore, determined primarily by the insect genotype [*13*].

Variation in adult insect cuticular hydrocarbon composition with age has been studied infrequently. Phillips et al. [*24*] reported that the total amount of the cuticular hydrocarbons of flies increased and then decreased with increasing age, but that the proportions of the individual components remained the same. Trabalon et al. [*25*] studied adult female blow flies, *Calliphora vomitoria* (Diptera: Calliphoridae), and found that the proportions of a few of their cuticular hydrocarbons did vary with age and that these changes corresponded to sexual maturation. These compounds varied within the first 24 hours post-emergence and between 72 and 92 hours post-emergence. The proportions of the majority of the cuticular hydrocarbons, however, remained unchanged as these flies aged.

The object of this study was to determine whether variation in the cuticular hydrocarbon composition occurs between different geographic populations of the black blow fly, *Phormia regina* (Meigen). We also were interested in determining whether there were any sex-specific differences in the surface hydrocarbon compositions, as these have been reported among Diptera [*17,26*]. Finally, we wanted to determine, through stepwise discriminant analysis, which components were important for population and sex differentiation.

Received for publication 29 July 1994; revised manuscript received 12 Sept. 1994; accepted for publication 12 Sept. 1994.

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This project was funded by The University of Georgia Agricultural Experiment Station.

Materials and Methods

Insect Collection and Maintenance

Phormia regina (Diptera: Calliphoridae) was chosen as the experimental species because it is widespread in the Holarctic region [*27*], in seasonal occurrence [*28*], and in its substrates [*27*]. Also, it can be trapped in the field and reared in the laboratory with ease.

Populations were obtained from three locations. A population from Lyle Grove (13 km southwest of Pullman, Whitman County, WA, elevation 646 m) was started from eggs that were taken from a porcupine carcass early in September, 1992. A population from Tucannon River (32 km northeast of Dayton, Columbia County, WA, elevation 1067 m) was started from adults that were collected near the river on July 25, 1992. The straight line distance between the two Washington populations was over 60 km. A population from Rensselaer (64 km north of Lafayette, Jasper County, IN, elevation 209 m) was started from specimens collected by N. H. Haskell in September, 1992, from a study pit containing swine carcasses.

Adult flies from each population were held at room temperature (23°C ± 4°C) in separate 45 × 45 × 45 cm metal-framed wire mesh screen cages. Water, a dry sugar/powdered milk mixture, and beef liver were provided because adults require dietary protein to develop eggs [*29*]. One slice of beef liver (approximately 50 g) was placed on damp filter paper and offered to adult flies from day 2 post-emergence until oviposition was first observed either on the liver or the filter paper.

Adult progeny of flies from each of these three populations were collected at 7 days post-emergence. Upon collection, these flies were frozen at −20°C, separated according to population and sex, and placed in 7 mL Solvent Saver™ glass scintillation vials. The vials were placed in an insulated container filled with dry ice and shipped to Athens, GA, via Federal Express for chemical analysis.

Cuticular Hydrocarbon Extraction

Insects were stored at −20°C until the hydrocarbons were extracted. Surface hydrocarbons were recovered by immersing each of 25 individual flies in redistilled hexane at room temperature for 60 seconds [*26*]. The extracts were concentrated to dryness under a stream of nitrogen and were then resuspended in 20 μL of hexane. Aliquots (5.0%) were analyzed by combined gas-chromatography/mass spectrometry (GC/MS). A Hewlett Packard 5890A gas chromatograph was equipped with a 25 m HP-1 cross-linked methyl silicone capillary column (0.2 mm internal diameter, 0.33 μm film thickness) with helium as the carrier gas. The initial oven temperature of 55°C was maintained for three minutes after injection and then was increased at a rate of 15°C/min until the temperature reached 305°C where it remained for 15 min. The column was connected to a Hewlett Packard 5970 mass selective detector, and mass spectra were recorded every 0.77 second at 70 eV. Components were characterized by their individual mass spectra, which were compared to those of standards, and were matched by computer search with an IBM-PC version of the NIST/EPA/NIH Mass Spectral Database. Equivalent chain lengths were determined by using standard *n*-alkanes (Sigma Chemical Co.) [*26,30*]. Quantitation of components was based on the integration of the total ion chromatograms that were corrected for response factors by using a standard for each class of cuticular hydrocarbon component [*31*]. Percent compositions of each component from the GC/MS analysis were averaged for each sex of each population, and standard deviations were calculated.

Canonical and Stepwise Discriminant Analyses

Results from the GC/MS analysis were subjected to discriminant function analysis in order to determine whether the cuticular lipid fractions could be used to discriminate flies by location and by sex [*32*]. Percent composition data for hydrocarbons that constituted a column sum of greater than or equal to five percent for all the flies were loaded into a 25 × 25 matrix (Lotus 123) (individuals × hydrocarbon components). Discriminant functions were calculated using the S-plus statistical analysis package (version 3.1) [*33,34*] running under SunOS 4.1.1 (UNIX) on a Sun Microsystems 4/280 computer. The discriminant analysis was performed three times, with the data grouped according to location, sex, then location and sex. Combined plots of either the first and second discriminant axes or the second and third discriminant axes were produced. In addition, stepwise discriminant analysis was performed in order to determine which compounds were most responsible for the separations in the discriminant function analyses [*35*]. These analyses were done using all flies by location and all flies by sex as the grouping variables.

Results

The cuticular hydrocarbons of *Phormia regina* adults were found by GC/MS analysis to be a mixture of *n*-alkanes, methyl-branched alkanes, and dimethyl-branched alkanes. The major hydrocarbons were *n*-pentacosane (Fig. 1, peak 11), 11- and 13-methylpentacosane (Fig. 1, peak 12), and *n*-heptacosane (Fig. 1, peak 19). Although the cuticular hydrocarbon patterns for flies from the three locations were similar, there were population-specific and sex-specific differences in the proportions of the components extracted (Table 1). For example, the amounts of 11-and 13-methyl-

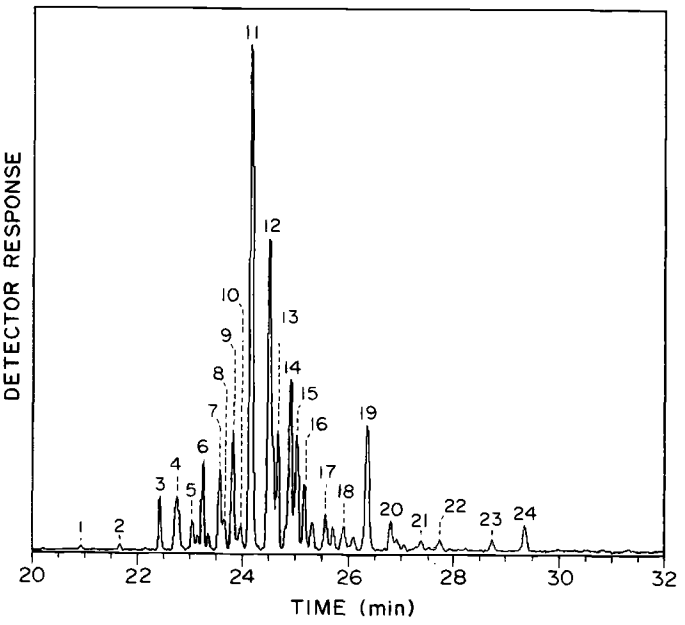

FIG. 1—*Total ion chromatogram of the cuticular hydrocarbons of an individual female black blow fly,* Phormia regina, *from Rensselaer, Indiana. Numbered peaks were identified by their mass spectra and are listed in Table 1.*

TABLE 1—*Cuticular hydrocarbons of adult flies from three geographic populations of* Phormia regina.

Peak[a]	ECL[b]	Component	Highly Variable Percent Composition	Components Most Responsible for Separation	
				By Population[c]	By Sex[d]
1		Unknown			
2		Unknown			
3	23.0	*n*-Tricosane			
4	23.3	9- & 11-Methyltricosane			
5	23.7	3-Methyltricosane			
6	24.0	*n*-Tetracosane			
7	24.3	12-Methyltetracosane		*	
8	24.5	6-Methyltetracosane		*	
9	24.6	2-Methyltetracosane	*	*	
10	24.8	X,12-Dimethyltetracosane			
11	25.0	*n*-Pentacosane	*		
12	25.3	11- & 13-Methylpentacosane	*		
13	25.5	5-Methylpentacosane			
14	25.7	3-Methylpentacosane			
15	25.9	5,13-Dimethylpentacosane		*	
16	26.0	*n*-Hexacosane			
17	26.3	13-Methylhexacosane		*	
18	26.6	2-Methylhexacosane			*
19	27.0	*n*-Heptacosane	*		
20	27.3	13-Methylheptacosane			
21	27.7	3-Methylheptacosane			
22	28.0	*n*-Octacosane			
23	28.6	2-Methyloctacosane			*
24	29.0	*n*-Nonacosane			

[a]Peak numbers correspond to those in Fig. 1.
[b]Equivalent Chain Length.
[c]Components most responsible for separation in discriminant analysis in Fig. 2.
[d]Components most responsible for separation in discriminant analysis in Fig. 3.

pentacosane and *n*-heptacosane in the surface lipids of female flies from Lyle Grove differed from each of the other groups of flies.

The four components which showed the most variation in average percent composition were 2-methyltetracosane, *n*-pentacosane, 11- and 13-methylpentacosane, and *n*-heptacosane (Table 1). There were greater differences in the percent composition of these four components in the cuticular hydrocarbons of the males than there were of the females for each population.

The results of the GC/MS analyses of the cuticular hydrocarbons of the 25 flies from the three populations were subjected to discriminant analysis in an effort to identify similarities between the cuticular hydrocarbon profiles of individual flies from each population. A plot of the second and third linear coefficients from this analysis using location as the grouping factor resulted in a pattern where the blow flies clustered in three distinct groups according to location (Fig. 2). There was no overlap between flies from the three populations, which were originally collected from two locations in the state of Washington and one location in Indiana.

The cuticular hydrocarbon analyses of the blow flies from the three populations were subjected to a discriminant analysis using sex as the discriminating factor. A plot of the first and second discriminant axes separated the 25 flies into two very distinct groups according to gender (Fig. 3). A discriminant analysis using the cuticular hydrocarbon data was then done using both location and sex as the discriminating factor. A plot of the first and second discriminant axes separated the 25 flies into six groups (Fig. 4). Males from the three locations, Lyle Grove, Washington, Tucannon River, Washington, and Rensselaer, Indiana, clustered into three distinct groups according to geographic location. The female blow flies also separated into three groups according to location, although the females from Lyle Grove were not well separated from the females from Indiana (Fig. 4).

Stepwise discriminant analysis was performed in order to determine which cuticular hydrocarbons were most responsible for separating populations of *P. regina*. The program identified 12-methyltetracosane, 6-methyltetracosane, 2-methyltetracosane,

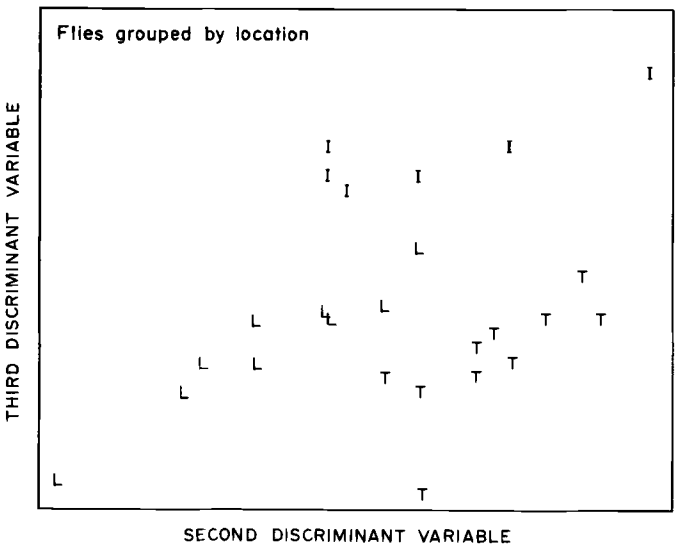

FIG. 2—*Plot of the second and third discriminant axes from a canonical discriminant analysis of the cuticular hydrocarbons of adult black blow flies,* Phormia regina, *using location as the grouping factor. Each letter represents one fly: flies were from Lyle Grove, Washington (L), Tucannon River, Washington (T), or Rensselaer, Indiana (I).*

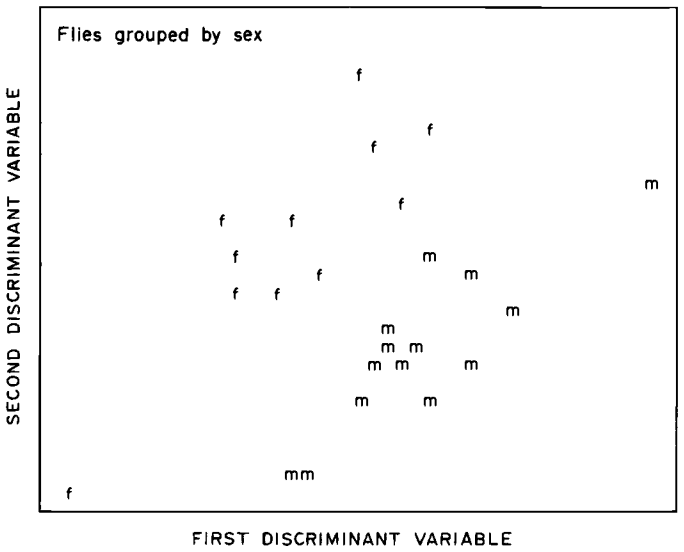

FIG. 3—*Plot of the first and second discriminant axes from a canonical discriminant analysis of the cuticular hydrocarbons of adult black blow flies,* Phormia regina, *using sex as the grouping factor. Each letter represents one fly: male (m) or female (f).*

5,13-dimethylpentacosane, and 13-methylhexacosane as being the hydrocarbons that were most important in the canonical discriminant analysis using location as the grouping variable (Table 1). A similar stepwise discriminant analysis showed that 2-methylhexacosane and 2-methyloctacosane were the components most important for the separation of black blow flies by sex.

Discussion

Cuticular Hydrocarbon Composition

This study provides a complete GC/MS characterization of the cuticular hydrocarbons of *P. regina* (Table 1). Our results agree with an earlier, partial characterization of *P. regina* surface hydrocarbons in which *n*-pentacosane and *n*-heptacosane were reported to be the major cuticular hydrocarbons of the black blow fly [*36*]. Louloudes et al. [*36*] examined the cuticular hydrocarbons of three species in the family Calliphoridae: *P. regina; Cochliomyia hominivorax* (Coquerel), a screwworm; and *Calliphora vicina* (Robineau-Desvoidy), a blow fly. Each fly species had a distinct cuticular hydrocarbon profile, but, in each case, *n*-alkanes ($C_{23}-C_{29}$) were major components. The hydrocarbons, 2-methyltetracosane, 2-methylhexacosane, and 2-methyloctacosane found in the surface lipids of *P. regina* (Table 1) are not commonly present in the cuticular lipids of Diptera [*13*]. However, Louloudes et al. [*36*] did report the presence of methyl-branched hydrocarbons with the methyl branch on C_2 for *P. regina, C. hominivorax,* and *C. vicina,* although the chain lengths of these hydrocarbons were not determined.

Alkenes were not detected in the cuticular hydrocarbons of *P. regina* (Table 1). In many dipteran species, alkenes are major components of the cuticular lipids, and they often serve as contact sex pheromones [*17,37*]. However, methyl-branched hydrocarbons also have been found to serve as contact sex pheromones in some Diptera [*38*]. Male and female *P. regina* have cuticular hydrocarbon profiles which can be distinguished by discriminant analysis (Fig. 3), and the components which are most responsible for this separation, 2-methylhexacosane and 2-methyloctacosane (Table 1), may serve as contact sex pheromones for this species. The fact that there was more variability in the hydrocarbon composition of *P. regina* males than of the females could be due to the fact that the cuticular hydrocarbons of the females may serve as contact sex pheromones [*17*]. Variation in these components in the cuticle of the female flies might have an adverse effect on reproductive pairing. Conversely, variation in male cuticular hydrocarbons might have an effect on female choice of a mate [*24*].

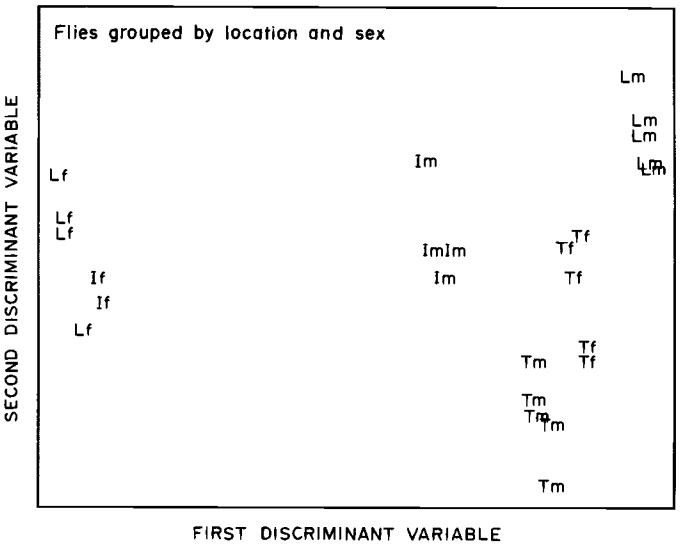

FIG. 4—*Plot of the first and second discriminant axes from a canonical discriminant analysis of the cuticular hydrocarbons of adult black blow flies,* Phormia regina, *using both location and sex as the grouping factor. Each fly is represented by two letters indicating location and sex: flies were from Lyle Grove, Washington (L), Tucannon River, Washington (T), or Rensselaer, Indiana (I), and were male (m) or female (f).*

Determination of Corpse Relocation

With the use of discriminant analysis, it was possible to distinguish separate geographic populations of the black blow fly, *P. regina,* on the basis of cuticular hydrocarbon profiles (Fig. 2). This result suggests that the analysis of blow fly cuticular hydrocarbons could be used to provide evidence of corpse relocation.

Distance and time from the death scene to the discovery site are key elements of death investigations [*39*]. The technique described here is applicable in those cases where evidence indicates that death scene and discovery site differ (for example, the lack of anticipated subcorpse faunal community). Immature blow flies could be recovered from a corpse, reared to adults, and the cuticular hydrocarbons could then be extracted and analyzed by GC/MS. A carcass could be placed at the site of corpse discovery in order to collect blow flies from that geographic area [*4–7*]. The cuticular hydrocarbons would be extracted from these flies and analyzed by GC/MS. The resulting hydrocarbon profiles would then be compared to those of flies recovered from the corpse. A distinct difference between the hydrocarbon profiles of the blow flies recovered from the corpse and those recovered from the local area, would be strong evidence of postmortem corpse relocation. As male and female blow flies of the same geographic population have distinct cuticular hydrocarbon profiles (Fig. 4), stronger evidence that blow flies recovered from a corpse did not come from the area where a corpse was found would be provided by separately comparing the cuticular hydrocarbon profiles of male and female blow flies from both groups of flies.

If a corpse has been moved from the death scene to the discovery site, it is very possible that blow flies from both locations will have oviposited on the body. Therefore, when blow flies of different developmental stages are recovered from a corpse that may have been moved, the flies should be maintained separately according to developmental stage, reared to adults, and the cuticular hydrocarbons extracted and analyzed. Variation in cuticular hydrocarbon profiles of these flies could provide evidence regarding relocation and the time when corpse movement took place.

The generally held belief that certain species of blow flies occur in urban or rural areas is problematic in suburban parkland and cemetery habitats. The technique described here might be used to verify the local source of blow flies associated with the corpse. Conversely, within a given species (here *P. regina*) perimeter individuals of each local population interbreed with those of adjacent populations, but the minimal interval between adjacent populations which is needed to show differences in the cuticular hydrocarbon profiles is unknown. Thus, in cases where short distances separate death and discovery sites, cuticular hydrocarbon differences could be misleading. However, when such differences do exist, then postmortem movement of the corpse might be concluded. Further studies are needed to determine how the boundaries for any local blow fly population might vary seasonally, how they are affected by topography, and, if they are to serve as a baseline for future investigations, whether or not they are stable over time.

Differentiation of insect populations by cuticular hydrocarbon analysis may be easier for nonentomologists to learn and apply than traditional identifications that use morphological characteristics [*40*]. In laboratories where a combined gas-chromatograph/mass spectrometer already is available, cuticular hydrocarbon analysis is comparatively inexpensive, and cuticular hydrocarbons are relatively easy to identify [*13,26,30*]. Forensic entomology has already proven to be very valuable in many different aspects of criminal investigations [*4*]. The ability to differentiate between geographic populations of blow flies by the analysis of cuticular hydrocarbon profiles also has the potential to become a valuable forensic tool.

Acknowledgments

We would like to thank Theresa Singer, Joy Layton and Julie Balsdon for assistance throughout this project. We thank Arden Lea, Head, Department of Entomology, University of Georgia and Lothar Tresp, Director, Honors Program, University of Georgia for their encouragement and support. We also thank an anonymous reviewer for helpful comments.

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