DOI: 10.2478/JAS-2014-0001 J. APIC. SCI. VOL. 58 NO. 1 2014

DE GRUYTER OPEN

Original Article

BODY-SURFACE COMPOUNDS IN BUCKFAST AND CAUCASIAN HONEY BEE WORKERS (*APIS MELLIFERA*)

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Received 26 November 2012; accepted 21 February 2014

Abstract

Body-surface chemical compounds were studied in 1-day-old nest workers and foragers both in Buckfast and Caucasian bees. The workers of these two age-castes were sampled twice in each of two consecutive years. Body-surface lipids were determined by means of gas chromatography, with a GCQ mass spectrometer. Protein concentrations and activities on the body surface were examined in bee cuticle rinsings obtained from worker bees according to the methods of Lowry, of Anson, and of Lee and Lin. Protease and protease inhibitor activities were determined. Polyacrylamide gel electrophoresis was performed. Caucasian bees, particularly foragers, had more lipids, but Buckfast bees (two age-castes) had more proteins on their body surfaces. A total of 17 alkane types (C17 - C33), 13 alkene types (C21 - C33), 21 esters (C12 - C32), and a phenol (C14) were detected in both races. Alkene C33 was detected only in Caucasian bees. More alkanes, esters, and phenols were found in Caucasian 1-day-old nest workers and foragers than in these age-castes of Buckfast bees. The protein concentration and protease inhibitor activities were lower in Caucasian bees that had higher protease activities. These values corresponded with specific numbers and widths of the electrophoretic bands.

Keywords: ***Apis mellifera*, chromatography (GC/MS), cuticle surface, lipid, protein, proteolysis.**

INTRODUCTION

The protective cuticle barrier in insects is the first line of defense against pathogens (Andersen, 1979). Many biochemical reactions proceed on the body surface of bees, e.g., sclerotization, melanization, and chitinization (Sugumaran, 2002; Merzendorfer and Zimoch, 2003; Evans et al., 2006; Burzyński et al., 2013). During these and other processes, which also occur in hemolymph or midgut, several chemical compounds are secreted on the chitinous carapace (Evans et al., 2006).

The first group consists of the lipids, which are responsible for both water repelling and transpiration, as well as functioning as pheromones or protecting bees against harmful environmental influences. The body-surface lipids usually contain hydrocarbons, waxy esters (long-chain alcohol esters of long-chain acids), primary alcohols, and fatty acids (Kolattukudy, 1968; Jackson and Baker, 1969; Plattner and Spencer, 1983; Howard and Lord, 2003; Buckner et al., 2009). These chemical compounds are also present on the surface of honey bee eggs (Arnold et al., 1996; Martin et al., 2002; Katzav-Gozansky

et al., 2003). Hydrocarbons are associated with lipophorins, which are multifunctional lipid carriers for phospholipids, diacylglycerols, and sterols as the cuticle evolves (Howard and Blomquist, 1982; Pho et al., 1996; Buckner et al., 2009). Phenols, which appear after ecdysis, also participate in cuticle layer formation. They are associated with the epicuticular filaments in both epicuticle and presumptive epicuticle (Locke and Krishnan, 2008). The surface esters, originally called "brood esters," are important sociochemicals within the colony (Keeling and Slessor, 2005).

The second group consists of the body-surface proteins, particularly proteases and protease inhibitors. To date, serine proteases, cysteine proteases, asparagine proteases, and metalloproteases have been found in bees (Grzywnowicz et al., 2009; Strachecka et al., 2011; 2012a,b,d,e). These enzymes safeguard the insects against infections and help maintain the homoeostasis of the apian body (Brownless and Williams, 1993; Gorman and Paskewitz, 2001), which depends, however, on the environment pollution level or application of common chemotherapeutics (Strachecka et al., 2010; 2012a,b,d).

Races of bees are discriminated on the basis of morphological and genetic markers, as well as hemolymph enzyme polymorphisms (De la Rua et al., 2009). Surface hydrocarbons help differentiate certain insect species, e.g., in *Nasutitermes* (*N. costalis* and *N. ephratae*; (Haverty et al., 1990) or honey bees (Africanized and European; Carlson and Bolten, 1983; Lavine et al., 1988; Lavine and Vora, 2005). An interesting hypothesis is that there is also interracial variability in apian cuticle chemical compounds. On the other hand, if the system of body-surface chemical compounds is susceptible to natural selection pressure, then it should be different in different races of bees. Therefore, the goal of the present research was to determine the body-surface chemical compounds and their interracial variability in 1-day-old nest workers and foragers in Buckfast versus Caucasian bees.

MATERIAL AND METHODS

The research was conducted on purebred Buckfast and Caucasian bees; 10 colonies in two consecutive seasons of 2010/2011 and 2011/2012 (10 colonies × 2 genotypes: Buckfast/Caucasian × 2 seasons). The colonies were headed by queens that had been instrumentally inseminated with the semen of single drones. Ten 1-day-old nest workers and ten foragers (different age-castes) were sampled from each colony once a season and pooled within each genotype and season to create 4 groups of 200 bees each; i.e., Buckfast foragers, Caucasian foragers, Buckfast 1-day-old workers, and Caucasian 1-day-old workers. Then, the body surfaces of 40 living bees from each of the 4 groups were analyzed by means of gas chromatography with a GCQ mass spectrometer (Thermo-Finnigan USA). Components (mostly lipids) were characterized by their individual mass spectra, which were compared to standards and matched by means of a computer search with an IBM-PC version of the NIST/EPA/NIH Mass Spectral Database.

Additionally, 3 samples comprising 10 1-day-old nest workers and 3 samples of 10 foragers each were collected twice a season from each colony within each genotype (2 worker age-castes × 3 samples × 10 colonies × 2 genotypes × 2 repetitions × 2 seasons). The material was frozen in sterile bags at -8°C and stored for 1 - 2 months. Then, the samples were successively refrozen and rinsed in 10 mL distilled water for 20 s to remove impurities. Proteins were not found in the rinsings using the Lowry method, as modified by Schacterle and Pollack (1973). Therefore, the rinsings were discarded. Subsequently, samples were shaken/rinsed for 4 min at 3400 rpm in 10 mL distilled water (hydrophilic proteins) and then in a 1% detergent solution (Triton X-100) in distilled water (10 mL). The solutions obtained from each sample were then divided into 3 portions, poured into 3 respective Eppendorf tubes, and frozen again at -40°C. The procedure produced a 2 mL sample that was used for determining protease and protease inhibitor activities (portion-a); 2 mL used for electrophoretic assays (portion-b); and 2 mL kept as reserve (portion-c).

The procedure resulted in a total of 2880 portions (480 samples × 2 rinsings × 3 portions). Portions were analyzed as follows: general protein content by the Lowry method, as modified by Schacterle and Pollack (1973); proteolytic activity in relation to the substrates (gelatin, hemoglobin, ovoalbumin, albumin, cytochrome C, casein) according to methods described by Anson (1938) and modified by Strachecka et al. (2011), with albumin selected for further study as the optimum substrate; activities of proteases according to the modified Anson (1938) method; and levels of natural protease inhibitors based on the Lee and Lin (1995) method.

Polyacrylamide gel electrophoresis was performed using portions-b, which had been previously lyophilized and then combined with 100 µL distilled water.

The following analyses were conducted: protease detection using the Laemmli (1970) method, and detection of inhibitors of asparagine and serine proteases using the modified Felicioli et al. (1997) method.
Multivariate general linear model (GLM) was carried out, allowing for the following factors: genotypes (Buckfast/Caucasian), age-castes, seasons, and colonies. Season and colony impact proved insignificant. Therefore, genotype and age-caste means were compared, using two-way ANOVA (genotypes × age-caste) and Tukey's test (SAS Institute Version 9.13., 2002-2003 license 86636).

RESULTS

Proteins and lipids were detected in both genotypes and both of the age-castes (Tab. 1). In general, Caucasian bees, particularly foragers, had more lipids, but Buckfast bees had more proteins on their body surfaces (Tab. 1 and 2). Four types of lipid compounds were found on bee cuticle: 17 alkane types (C17 - C33), 13 alkene types (C21 - C33), 21 esters (C12 - C32), and a phenol (C14) (Fig. 1 and 2). Alkene C33 was detected only in Caucasian bees, and the remaining chemical compounds were present on worker cuticles in both bee genotypes. The compound quantities were, however, different between Caucasian and Buckfast bees. More esters were found in Caucasians, but more alkenes were detected in Buckfast workers, independently of the age-caste (Tab. 1; Fig. 1 and 2). Consequently, there were no significant age-caste × breed interactions ($P>0.1$). The Caucasian foragers had more body-surface alkanes and phenols than the Buckfasts, which was not observed, however, in 1-day-old nest workers. Consequently, there were significant breed × age-caste interactions ($P<0.01$) in this case.
Although the protein concentration (Tab. 1) was lower in Caucasian bees, they had higher protease activities than the Buckfasts. These differences were observed both in foragers and 1-day-old nest workers. On the other hand, the body-surface protease inhibitors had similar activities in foragers in both genotypes whereas in 1-day-old Buckfast workers, the inhibitors were more active than in the Caucasians. Thus, Caucasian bees had a greater capacity for proteolysis than for its inhibition.
Foragers had lower esters and more alkenes than 1-day-old nest workers. Otherwise, these age-castes did not differ, and genotypes had no significant influence in the case of the lipid compounds. On the other hand, 1-day-old workers had significantly larger protein concentration and protease activities but lower protease inhibitor activities. These relationships were evident in both genotypes. Thus, the age caste markedly influenced the protein concentration and the cuticle proteolytic system activity.
The electrophoretic images (Tab. 3, Fig. 3) correspond with the above results because higher band numbers and larger band widths were found at higher protein concentrations/higher protease activities/higher protease inhibitor activities.

DISCUSSION

Our studies have shown that the outermost layer of the apian cuticle is composed of alkanes, alkenes, esters, phenols, and proteins but that the amounts of some of these chemical compounds as well as body-surface enzyme activities differ in Buckfast and Caucasian workers. Caucasian bees have more lipids, which is particularly apparent in foragers, but Buckfast bees (two age-castes) have more proteins. This difference could be accounted for by the large amounts of propolis gathered by the Caucasian bees. Consequently, there is chemical interracial variability in apian body surface. Worth mentioning are esters, alkenes, and proteins, including proteases, because these chemical compounds had different amounts/activity in Buckfast and Caucasian workers independently of age-caste. Surface hydrocarbons have been used for species discrimination in *Nasutitermes* (Haverty et al., 1990), and Africanized and European honey bees (Carlson and Bolten, 1983; Lavine et al., 1988; Lavine and Vora, 2005). Our results showed that these substances also may be taken into consideration for race/breeding line discrimination (De la Rua et al., 2009) or the interpretation of evolutionary *A. mellifera* race histories (Chapuisat, 2014). Foragers appeared to be the best material for the interracial variability studies with the use of lipids; we found distinct interracial differences in all lipid compound groups in this age-caste.
The higher cuticle lipid fraction in Caucasian bees could be connected with a higher water content in their tissues (Hadley, 1981; Muszyńska, 1988), which may result in lower resistance during overwintering in bees containing more water (Muszyńska, 1988). This concept corresponds with Olszewski's (2007) findings that Buckfasts are more resistant than Caucasians to adverse winter weather conditions. Thus, the lipid fraction concentration could be a factor in the natural selection of overwintering bees and therefore might have been modified during the evolution of bees.
C33 alkanes seemed to be of particular importance

Table 1.
Chemical compounds detected on the cuticles of 1-day-old workers and foragers in Buckfast (Bcf) and Caucasian (Cau) bees

		Bcf	Cau	Overall mean ± SD
	Compound type	Mean ± SD	Mean ± SD	
1-day-old workers	Line alkanes, branched alkanes, methyl alkanes (%)	1.38 ± 0.07	1.46 ± 0.02	1.42 ± 0.05
	Alkenes (%)	1.30^{a} ± 0.07	1.03^{b} ± 0.01	1.30^{n} ± 0.04
	Esters (%)	2.75^{b} ± 0.15	3.53^{a} ± 0.05	2.63^{m} ± 0.09
	Phenols (%)	0.18 ± 0.01	0.19 ±0.01	0.11 ± 0.06
	Proteins (concentration C; mg/mL)	0.64 ± 0.04	0.48 ± 0.12	0.56^{m} ± 0.08
	Proteases (activity; as U/mg)	11.68^{b} ± 0.05	29.97^{a} ± 0.36	20.82^{m} ± 0.20
	Protease inhibitors (activity; as U/mg)	6.70^{a} ± 0.17	2.57^{b} ± 0.19	4.64^{n} ± 0.18
Foragers	Line alkanes, branched alkanes, methyl alkanes (%)	1.36^{b} ± 0.08	2.05^{a} ± 0.03	1.41 ± 0.09
	Alkenes (%)	1.86^{a} ± 0.10	1.67^{b} ± 0.02	1.86^{m} ± 0.06
	Esters (%)	2.14^{b} ± 0.12	2.43^{a} ± 0.04	2.06^{n} ± 0.07
	Phenols (%)	0.05^{b} ± 0.01	0.13^{a} ± 0.01	0.09 ± 0.03
	Proteins (concentration C; mg/mL)	0.36^{a} ± 0.02	0.07^{b} ± 0.01	0.22^{n} ± 0.02
	Proteases (activity; as U/mg)	7.03^{b} ± 0.03	14.04^{a} ± 0.28	10.53^{n} ± 0.15
	Protease inhibitors (activity; as U/mg)	7.99 ± 0.03	7.97 ± 0.38	7.98^{m} ± 0.21
Overall mean ± SD	Line alkanes, branched alkanes, methyl alkanes (%)	1.37^{a} ± 0.11	1.75^{b} ± 0.07	
	Alkenes (%)	1.58^{a} ± 0.12	1.35^{b} ± 0.11	
	Esters (%)	2.45^{a} ± 0.14	2.98^{b} ± 0.08	
	Phenols (%)	0.12 ± 0.09	0.16 ± 0.06	
	Proteins (concentration C; mg/mL)	0.50^{a} ± 0.03	0.28^{b} ± 0.09	
	Proteases (activity; as U/mg)	9.36^{a} ± 0.17	22.00^{b} ± 0.22	
	Protease inhibitors (activity; as U/mg)	7.34^{a} ± 0.25	5.27^{b} ± 0.23	

a, b – Differences are statistically significant for comparisons between Bcf and Cau means (P≤0.05). m, n –Differences are statistically significant for comparisons between overall means calculated for foragers and 1-day-old workers (P≤0.05); SD – standard deviation.
Shaded fields relate to values significantly higher than unshaded values.
The amounts of non-protein chemical compounds were calculated from the peak sizes in GC/MS. Protease and protease inhibitor activities were calculated from the Anson (1938) formula.

Table 2.

The percentage of individual compounds in the lipid fraction in 1-day-old Buckfast/Caucasian workers and Buckfast/Caucasian foragers (Bcf/Cau)

Compound type	Foragers		1-day-old workers	
	Bcf	Cau	Bcf	Cau
Line alkanes, branched alkanes, methyl alkanes	25	31	31	29
Alkenes	33	24	21	17
Esters	42	45	48	54
Phenols	0.05	0.13	0.18	0.19

Table 3.

PAGE zymography of proteins and the activity of proteases and protease inhibitors on the body surface of 1-day-old workers and foragers in Buckfast (Bcf) and Caucasian (Cau) bees

		Foragers				1-day-old workers			
		Bcf		Cau		Bcf		Cau	
Substance	pH	Band number	OD	Band number	OD	Band number	OD	Band number	OD
Proteins	2.4	5	0.21	4	0.25	4	0.26	4	0.23
	7.0	6	0.31	5	0.23	6	0.21	5	0.21
	11.2	5	0.35	4	0.28	6	0.28	6	0.23
Proteases	2.4	5	0.32	8	0.39	9	0.29	9	0.32
	7.0	3	0.30	6	0.35	4	0.33	6	0.43
	11.2	4	0.21	5	0.22	4	0.26	6	0.29
Protease inhibitors	2.4	4	0.21	4	0.11	4	0.23	2	0.18
	7.0	4	0.26	4	0.24	6	0.27	5	0.21
	11.2	4	0.30	4	0.29	4	0.16	3	0.15

OD – width of the bands (mm).

The results for protease activities in PAGE zymography are arithmetic means of the results obtained for Bcf or Cau foragers and 1-day-old Bcf or Cau workers.

for bee race discrimination because they were present only in Caucasian bees. C33 alkanes are biologically very active. They are identified as the major components of the homosexual courtship-stimulating pheromone blend in *Drosophila melanogaster* (Schaner et al., 1989). These compounds are responsible for inducing male fly copulatory behavior in *Stomoxy calcitrans* (Sonnet et al., 1979). High amounts of C33 alkanes were detected on worker body-surfaces, but amounts were low in queens in *Friesella schrottkyi*, and these compounds are elements of the aggressive pheromone (Nunes et al., 2010). Caucasian bees have a dysfunction of some type of the pheromone that triggers reproduction of varroa mites (Stort and Rebustini, 1998; Strachecka et al., 2012c, 2013), but they have more alarm pheromones than Buckfast bees (Harmon and VanEnglespdorp, 2004). Therefore, C33 alkenes may affect the specific behavior of Caucasian bees, which should be studied in the future.

Chain linear and/or branched alkanes, methylalkanes, and alkenes are common components of many insect surface lipids and in some species constitute more than 90% of this total fraction (Hadley, 1981). Our studies showed that this percentage was lower in bees, ranging from 52% to 58% in Buckfast bees and from 46% to 55% in Caucasian bees in the total of this fraction. Honeybees and dragonflies contain up to 58% of cuticular wax esters and phenols, having a total of 36–50 carbon atoms (Jacob and Hanssen, 1979; Patel et al., 2001). In our studies, Caucasian and Buckfast bees had respectively 45

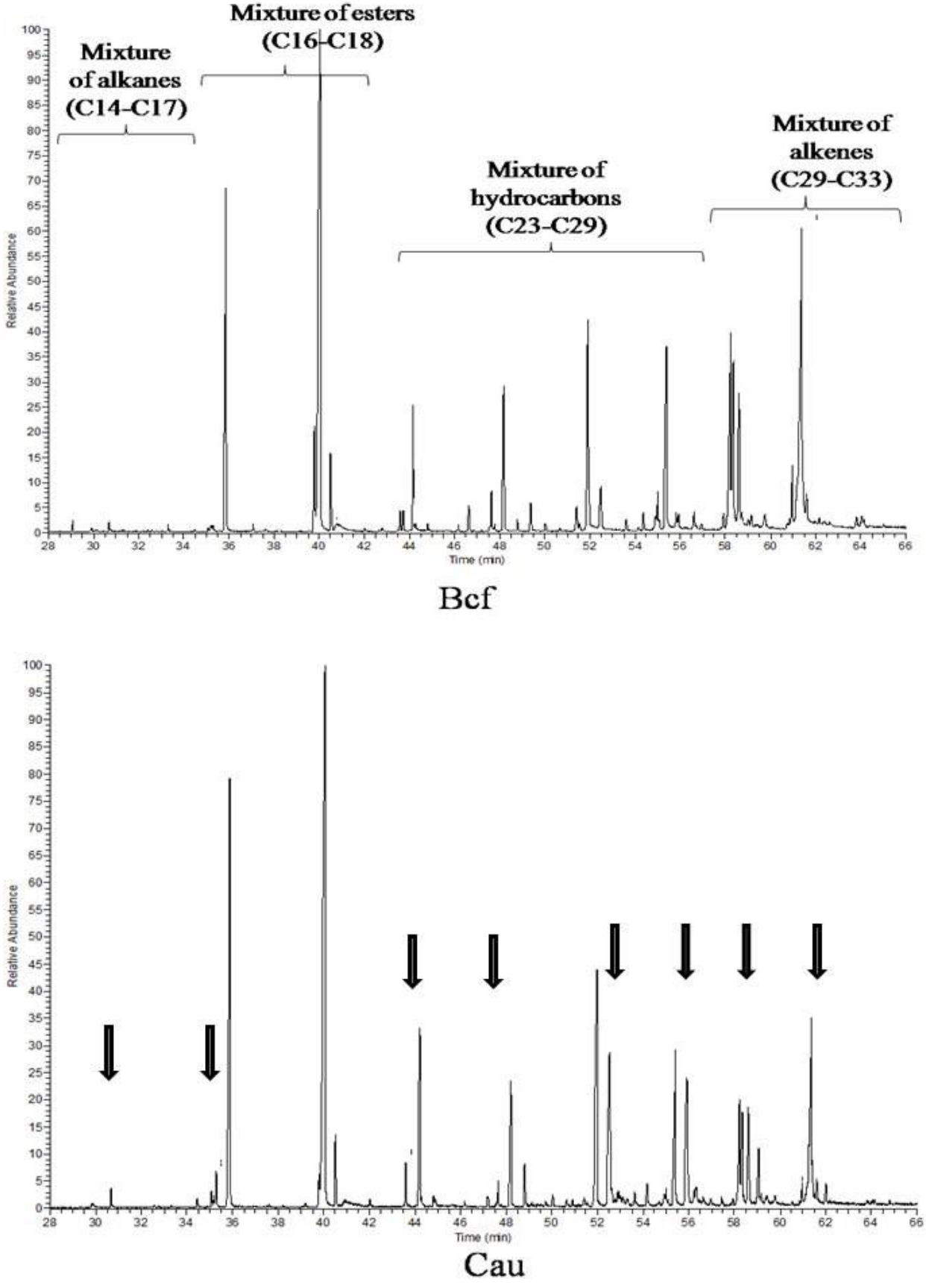

Fig. 1. Chromatograms of the forager cuticle chemical compounds in Buckfast (Bcf) and Caucasian (Cau) bees. Arrows indicate differences between Bcf and Cau in numbers or sizes of peaks.

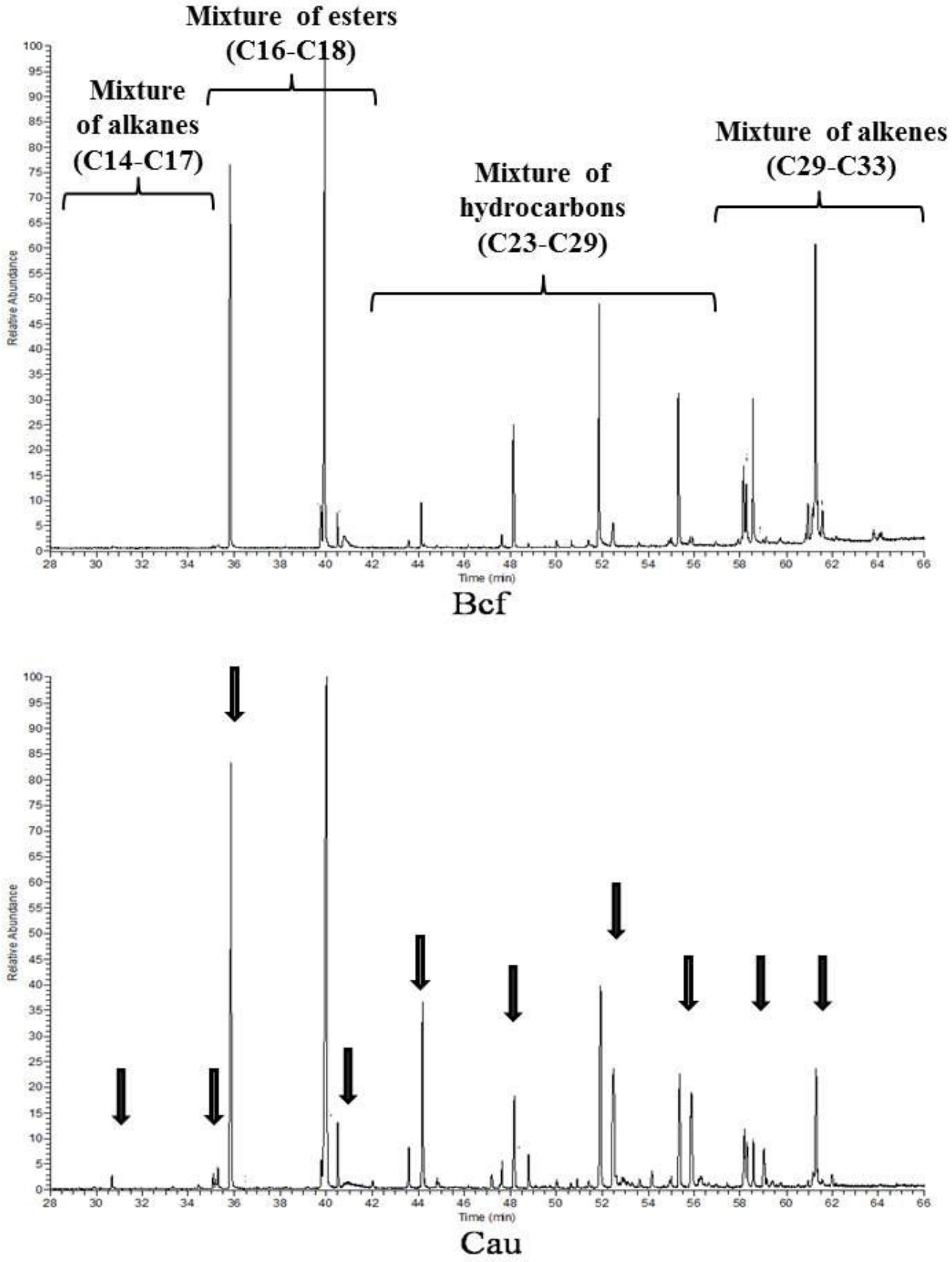

Fig. 2. Chromatograms of the 1-day-old worker cuticle chemical compounds in Buckfast (Bcf) and Caucasian (Cau) bees.
Arrows indicate differences between Bcf and Cau in numbers or sizes of peaks.

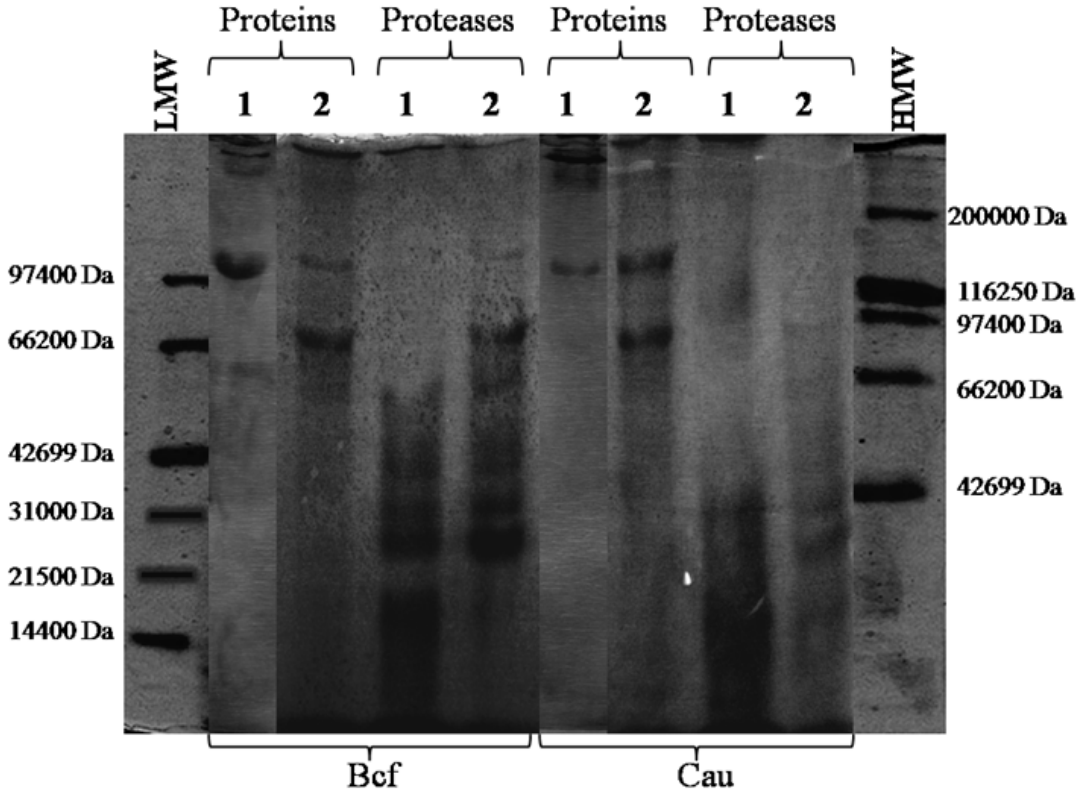

Fig. 3. Electrophorogram of cuticle protein concentrations and protease activities in Buckfast (Bcf) and Caucasian (Cau) 1-day-old workers (1) and foragers (2). LMW – low-molecular standard; HMW – high-molecular standard.

- 54% and 42 - 48% of C12 - C32 esters on their cuticles. The esters, as pheromone constituents, could play a role in the competition of bees for the same food resources and in trail scenting (Jarau et al., 2006). These results do not correspond, however, with the low competitive abilities of the Caucasian foragers observed by Paleolog (2009). Our studies showed that Caucasian bees had more esters. They are a race that monitors a relatively larger area, and the insects move faster than Buckfasts from one nectarous plant to another (Konopacka, 1999; Borsuk and Olszewski, 2010; Olszewski et al., 2013). Buckfasts had higher cuticle-protein concentration, but Caucasian bee cuticles had a greater capacity for proteolysis and lower capacity for its inhibition. This proteolytic system is an element of cuticle-associated anti-pathogen resistance (Evans et al., 2006; Zou et al., 2006). How these results relate to the high resistance of Buckfasts to some pathogens (Brother Adam, 1983, 1993; Österlund, 1999) is difficult to say. Additional studies are needed.

CONCLUSIONS

Body-surface protein concentration and proteolytic activity may be taken into consideration during breed discrimination or studies of the evolutionary histories of bees because of distinct, age-caste–independent, interracial variability in these chemical compounds. Lipids also might be considered, but only in the forager age-caste. The C33 alkanes are the most appropriate chemical compounds with which to begin any such analysis.

The interracial variability in the lipid and protein cuticle cover could be caused by natural selection pressure.

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