

1 **Geographical variation of cuticular hydrocarbon profiles of adult**  
2 **flies and empty puparia amongst three populations of *Calliphora***  
3 ***vicina* (Diptera: Calliphoridae)**

4 **Canan Kula<sup>1,\*</sup>, Jens Amendt<sup>2</sup>, Falko P. Drijfhout<sup>3</sup> and Hannah E. Moore<sup>1</sup>**

5 <sup>1</sup>Cranfield Forensic Institute, Cranfield University, Defence Academy of the United Kingdom, Shrivenham,  
6 Wiltshire SN6 8LA, UK

7 <sup>2</sup>Institute of Legal Medicine, University Hospital Frankfurt/Main, Goethe-University, 60323 Frankfurt/Main,  
8 Germany

9 <sup>3</sup>Chemical Ecology Group, School of Chemical and Physical Science, Keele University, Keele ST5 5BG, UK

10 \*Correspondence: [c.kula@cranfield.ac.uk](mailto:c.kula@cranfield.ac.uk)

11 **Abstract**

12 Blowflies (Diptera: Calliphoridae) are of great importance in forensic entomology and in  
13 determining the minimum post-mortem interval, as they may be the first group of insects to  
14 colonise decomposing remains. Reliable species identification is an essential prerequisite.  
15 Classically, morphological characters or DNA sequences are used for this purpose. However,  
16 depending on the species and the condition of the specimen, this can be difficult, e.g. in the case  
17 of empty fly puparia. Recent studies have shown that cuticular hydrocarbon (CHC) profiles are  
18 species-specific in necrophagous taxa and represent another promising tool for identification.  
19 However, the population-specific variability of these substances as a function of e.g. local  
20 climatic parameters has not yet been sufficiently investigated. The aim of this study was to  
21 determine the geographical variation of CHC profiles of the blowfly *Calliphora*  
22 *vicina* depending on different countries of origin. Flies were reared in the UK, Germany and

23 Turkey in common garden experiments under ambient conditions. CHC profiles of the resulting  
24 adult flies and their empty puparia were analysed using gas chromatography-mass  
25 spectrometry. Data were visualised by principal component analysis and clustered by  
26 population. The populations of the UK and Germany, both having similar climates and being  
27 geographically close to each other, showed greater similarities in CHC profiles. However, the  
28 CHC profile of the Turkish population, whose climate is significantly different from the other  
29 two populations, was very different. Our study confirms the high potential of CHC analysis in  
30 forensic entomology but highlights the need to investigate geographical variability in chemical  
31 profiles.

32

33 *Keywords:*

34 Calliphoridae, adult fly, empty puparia, cuticular hydrocarbons, GC-MS, Turkey, Germany,  
35 United Kingdom

## 36 **Introduction**

37 Calliphoridae (Diptera) is a significant family in the field of forensic entomology due to the fact  
38 that they are among the primary insects colonised on human bodies. (Benecke, 2001; Amendt  
39 et al., 2004). Forensic investigators consider them to be crucial evidence regarding the time and  
40 location of death (Catts and Goff, 1992; Amendt et al., 2007; Gennard, 2007; Moore et al.,  
41 2017a). A minimum post-mortem interval (minPMI) can be determined by using the arrival of  
42 adult insects and the resulting colonisation patterns in conjunction with the age estimation of  
43 the immatures developing on the dead body (Michaud and Moreau, 2009; Pechal et al., 2014).  
44 Accurate species identification is important for estimating a minPMI, as the duration of the life  
45 cycle and the ageing process varies by species (Moore et al., 2013; Paula et al., 2017). For an

46 accurate calculation, reference growth data obtained in the laboratory are used for the respective  
47 species (Moore and Shemilt, 2018). However, a potential source of error in determining  
48 minPMI is ignoring the fact that data from the laboratory may not reflect the developmental  
49 adaptations of the local population of interest. Therefore, Tarone et al. (2011) emphasised the  
50 need to determine how ecological conditions influence physiological responses and can lead to  
51 differences in growth rate between populations of the same species that exist many hundreds or  
52 thousands of kilometres apart, e.g. on a latitudinal scale. Hwang and Turner (2009) suggested  
53 the existence of local adaptations that can be seen on a small scale that different levels of  
54 urbanisation may cause for the forensically important blowfly *Calliphora vicina* (Robineau-  
55 Desvoidy, 1830), but, e.g. Limsopatham et al. (2018) found similar larval growth rates for this  
56 species from the UK and German populations at different temperatures during the major part  
57 of development.

58 Another important aspect of forensic entomology is providing information whether the crime  
59 was committed at the place of discovery. If a corpse is colonised with insects typical of a  
60 different habitat or geographical region, this may indicate that the corpse has been transported.  
61 However, Charabidze et al. (2017) rightly point out that since the majority of European  
62 necrophagous species have wide distribution ranges that cover many countries, sampling non-  
63 native species inside European regions is improbable. Additionally, although each species has  
64 a preferred ecological environment, individuals from the species may sometimes be found  
65 outside of the chosen range. Hence, it is difficult to link a certain species with a particular  
66 location or environment.

67 For proving population-specific differences and habitat adaptations, which could be linked to a  
68 change in development, the differentiation or assignment of such clusters would be desirable.  
69 For this purpose, e.g. molecular biological and morphological analyses are suitable. (Kranz et  
70 al., 2017). Zhou et al. (2020) investigated phylogenetic relationships among Calliphoridae

71 species using DNA-based sequence data and measured the degree of genetic variation.  
72 Landmark-based geometric morphometric analysis has also recently been employed in  
73 entomology. However, DNA-based techniques require molecular biology experience and  
74 extensive equipment to perform gene sequencing and so far do not reveal any evidence of a  
75 consistent difference between populations. While morphometric data collection is manageable,  
76 statistical and computer-assisted analysis requires skills and experience. Also, different  
77 geographical populations could not separate from each other (Limsopatham et al., 2018). An  
78 alternative technique that has the potential to distinguish the variations in various populations  
79 of the same forensically important species is cuticular hydrocarbon (CHC) analysis.

80 For a few years now, the chemistry of the insect cuticle has been studied as a new tool in  
81 forensic entomology. The cuticle is coated with a waxy layer consisting of hydrocarbons, fatty  
82 acids, and alcohols(Gibbs, 1998a). The primary function of the cuticular layer is to maintain  
83 water balance and prevent desiccation; as insects adapt to new habitats with different  
84 temperatures and climate types, the composition of the layer changes (Chung and Carroll,  
85 2015). The hydrocarbons are found as saturated (alkanes) and unsaturated forms (alkenes and  
86 alkynes), which can have one or more methyl groups. Unsaturated forms can contain one, two,  
87 or three double bonds along the chain length (Moore et al., 2013). Long-chain hydrocarbons  
88 with higher boiling points provide more efficient protection at higher temperatures than methyl-  
89 branched and unsaturated hydrocarbons with lower boiling points (Drijfhout et al., 2010).  
90 However, methyl-branched and unsaturated hydrocarbons are present in the epicuticular layer  
91 even though they reduce the ability of the protective barrier, as they are required for signal  
92 exchange during chemical communication in nestmate recognition and sex determination  
93 (Toolson, 1984). need more mobility and flexibility, especially in the early immature stages.  
94 To increase the flexibility, branched methyl alkanes and alkenes with lower melting points are

95 needed. As the larvae grow and develop, especially in the post-feeding stage, mobility and  
96 flexibility decrease and longer chain hydrocarbons are needed (Drijfhout et al., 2010).

97 The biophysical properties of surface epicuticular lipids also vary among the same species, and  
98 this variation is probably due in part to genetic and environmental factors (Gibbs et al., 1991).  
99 Noorman and Den Otter (2002) investigated the effects of relative humidity, temperature, and  
100 population density on the CHC profiles of adult *Musca domestica* L. (house flies) and showed  
101 that the CHC profile was affected by relative humidity and temperature, and the change was  
102 traceable.

103 The pupae are immobile, and protection against dehydration is vital; when the larvae face  
104 threats such as sunlight or lower relative humidity, they can move away, but pupae cannot  
105 escape exposure (Paula et al., 2017). Therefore, a cuticle that provides effective waterproofing  
106 to the pupae may avoid fatal impacts. Toolson (1982) examined the effects of larval and  
107 puparial thermal regimes on transcuticular water loss rates and epicuticular composition in adult  
108 *Drosophila pseudoobscura* (fruit flies) and showed that the temperatures exposed during the  
109 puparial period are influential in shaping the cuticular profiles of adult flies. Toolson and  
110 Hadley (1979) examined the cuticle permeability and lipid composition of *Centruroides*  
111 *sculpturatus* Ewing 1928 (scorpions), which they collected in the summer and winter seasons,  
112 and revealed the seasonal effect on the structure. Kruger and Pappas (1993) demonstrated the  
113 geographic variation of the CHC profile among fourteen populations of *Aedes albopictus*  
114 (Skuse, 1895) (mosquitoes). Chung and Carroll (2015) investigated how ecological variation in  
115 insect CHC profiles can lead to differentiation in mating signals and reproductive isolation.  
116 They noted that the evolution of CHC profiles might be the best method for understanding  
117 ecological speciation.

118 Previously, geographical differences were studied by examining the CHC profiles of beetles  
119 (Chown et al., 2011; Hadley, 1978; Howard et al., 1995), wasps (Espelie et al., 1990; Michelutti  
120 et al., 2018), cockroaches (Brown et al., 2000; Everaerts et al., 1997), grasshoppers (Gibbs et  
121 al., 1991; Tregenza et al., 2000), sandflies (Kamhawi et al., 1987), ants (Martin et al., 2008;  
122 Menzel et al., 2017), mosquitoes (Anyanwu et al., 2000; Kruger et al., 1991; Kruger and Pappas,  
123 1993; Rosa-Freitas et al., 1992) and fruit flies (Bontonou et al., 2013; Chung and Carroll, 2015;  
124 Ingleby et al., 2013; Markow and Toolson, 2013; Rajpurohit et al., 2017; Rouault et al., 2001,  
125 2004). However, studies on blowfly species are rather limited (Byrne et al., 1995; Moore et al.,  
126 2022; Paula et al., 2017, 2018, 2020).

127 This study aims to investigate the variation between the chemical profiles of the forensically  
128 important blowfly, *Calliphora vicina* (Diptera: Calliphoridae), and their empty puparia reared  
129 outdoors in different habitats in the UK, Germany and Turkey. It will also determine if any  
130 potential local adaptations have an influence on their chemical profiles. Differentiation will be  
131 based on qualitative and quantitative differences in CHC profiles.

## 132 **Materials and methods**

### 133 **Insect materials**

134 *C. vicina* were obtained from three locations: Swindon/United Kingdom (51°36'23.2"N  
135 1°38'06.5"W), Frankfurt/Germany (50°05'39.2"N 8°39'52.4"E) and Ankara/Turkey  
136 (39°52'24.9"N 32°43'48.7"E) (see in Figure 1). Straight-line distances are over 740 km between  
137 populations of the UK and Germany, over 2190 km between populations of Germany and  
138 Turkey, and over 2930 km between populations of the UK and Turkey. The rearing of adult  
139 flies and creating of the empty puparia were carried out separately in the relevant countries. The  
140 adult flies were collected from the wild in the summer months and placed outdoors in rearing

141 cages under ambient environmental conditions and were supplied with blood, sugar, water and  
142 milk powder. Fresh lamb liver was used as an oviposition medium. Once fly eggs were laid on  
143 the oviposition medium, the Petri dish containing the eggs and meat was transferred into small  
144 rearing boxes. The boxes were also placed in outdoor cages. During the pupariation period, the  
145 cages were checked daily. On the first day of metamorphosis, the first generation of adult flies  
146 and their empty puparia were collected and transported to the laboratory in the relevant  
147 countries for the extraction process.

### 148 **Sample preparation**

149 The liquid extraction method described by Moore et al. (2017a) was chosen for CHCs  
150 extraction. For the extraction of CHCs, a non-polar solvent (hexane) was used. The extractions  
151 were carried out ten times (n = 10) without gender separation for each population. For adult fly  
152 and their empty puparia extractions, a specimen was placed into a GC vial with hexane,  
153 ensuring that the specimen was fully submerged (500  $\mu$ L for adult flies, 350  $\mu$ L for empty  
154 puparia) and left for 10-15 minutes. The hexane extract containing the CHC profile was directly  
155 transferred to a clean vial. Then, the hexane was left to evaporate until the extract could be  
156 transferred to 300  $\mu$ L glass inserts and left to dry down completely. Finally, all samples were  
157 stored in the refrigerator at 4°C until they were required for analysis. The dried extract was then  
158 reconstituted in 30  $\mu$ L of hexane for GC-MS analysis.

### 159 **Chemical analysis of extracts**

160 Chemical analysis of extraction samples prepared in the laboratories of the relevant countries  
161 was performed in the same laboratory on the same Agilent Technologies 6890N Network GC  
162 system with a split/splitless injector at 250°C, a Restek Rxi-1MS capillary column containing  
163 an SP of 100% Polydimethylsiloxane (30 m x 0.25 mmID, 0.25  $\mu$ m film thickness) coupled to

164 an Agilent 5973 Network Mass Selective Detector. The GC was connected to a computer, and  
165 the data was processed with Agilent Chemstation software. Elution was carried out with helium  
166 at 1 mL/min. The oven temperature was programmed to be held at 50°C for 2 minutes, then  
167 ramped up to 200°C at 25°C/min, then from 200°C to 260°C at 3°C/min and finally from 260°C  
168 to 320°C at 20°C/min where it was held for 2 minutes. The mass spectrometer was operated in  
169 Electron Ionisation at 70 eV, scanning from 40-500 amu at 1.5 scans/s. CHCs were identified  
170 using a library search (NIST08).

## 171 **Statistical analysis**

172 Datasets from the chromatograms were initially interpreted using PCA to facilitate the  
173 visualisation of trends that might be found. For more details, the reader is referred to Moore  
174 and Drijfhout (2013; 2015). PCA has already been used by the authors in previous studies  
175 (Moore et al., 2013, 2016, 2017a, 2017b), and the same experimental design was used for this  
176 study. CHCs with a percentage peak area of less than 0.5% were excluded for statistical  
177 analysis. For PCA, peak areas on chromatograms were identified using Agilent Chemstation  
178 software, focusing on only CHCs. All data were normalised before the PCA calculation. In  
179 most experiments, normalisation is accomplished by dividing the absolute abundance  
180 (concentration) of each component by the total abundance of all compounds, and a relative  
181 abundance is calculated, therefore minimising the effects of the high within-treatment variation  
182 (Moore and Drijfhout, 2015). The n-alkanes used for this study ranged from C23:H to C31:H.  
183 The NCSS 2020 software was used to construct the 3D graphic plane using the dataset acquired  
184 by PCA.

## 185 **Results**

### 186 **CHC profile analysis and chemical identification**

187 **Adult flies**

188 A total of 46 CHC compounds were identified in the CHC profiles of the adult *C. vicina*  
189 collected from both the UK and Germany, and 31 CHCs in those collected from Turkey. The  
190 CHCs detected in the chromatograms are given in Table 1, with the total percentage peak area  
191 and standard deviation of each compound. All three populations had the same number of n-  
192 alkanes. One of the most noticeable differences between the three populations was the number  
193 of branched methyl alkanes and alkenes. The Turkish population showed fewer branched  
194 methyl alkanes than the UK and German populations. While one alkene compound was detected  
195 in the Turkish population, the alkenes detected in the UK and German populations were  
196 excluded from the comparison because of insufficient concentrations. In the CHC profiles, n-  
197 alkanes with odd-chain-length (OLA) were detected in higher concentrations than those with  
198 even-chain-length n-alkanes (ELA) (Roux et al., 2008). C27:H (Table 1, compound 20) was the  
199 dominant n-alkane for all three populations, and its percentage concentration was similar in all  
200 the populations. The next predominant compound with a higher concentration in all profiles  
201 was C29:H (Table 1, compound 35), and its percentage concentration in the Turkish population  
202 was 4% higher than in the UK and German populations, where the percentage concentrations  
203 of C29:H were similar.

204 When looking at the difference between populations, it was found that 9-MeC25 (Table 1,  
205 compound 7) was detected exclusively in the Turkish population, 3-MeC25 (Table 1, compound  
206 11) only in the UK, and 5-MeC26 (Table 1, compound 16) only in the German population.  
207 11+13-MeC27 (Table 1, compound 21) was the most predominant branched methyl alkane in  
208 all three populations. However, differences in its concentration differed between populations.  
209 Its concentration in the UK and Germany populations was almost the same but lower than in  
210 the Turkish population. In the UK and German populations, which have almost similar CHC  
211 profiles, 3-MeC27 (Table 1, compound 27) and 11+13-MeC29 (Table 1, compound 37) also

212 had higher but different quantitative concentrations than other branched methyl alkanes, while  
213 7-MeC29 had the second-highest concentration in the Turkish population.

214 Figure 2 shows the GC chromatograms of adult flies of *C. vicina* from the UK, German and  
215 Turkish populations. The CHC profiles were extracted on the first day immediately after  
216 eclosion. While the chemical profiles of the UK and German populations were quite similar,  
217 the number and concentration of CHCs with a shorter carbon chain were much higher in the  
218 Turkish population than in the other populations. In addition, there were CHCs with higher  
219 retention times (also known as longer chain CHCs) in the Turkish population that were not  
220 found in the UK and German populations.

221 Figure 3 compares the total concentrations of CHCs detected in the chemical profiles of adult  
222 flies against the populations. The concentrations of branched methyl alkanes were significantly  
223 higher than the concentrations of n-alkanes. The Turkish population had greater total  
224 concentrations of n-alkanes than the other populations, although all populations had the same  
225 total number of n-alkanes found in their profiles. The UK and German populations already had  
226 a higher number of branched methyl alkanes, and their total concentration was higher than that  
227 of the Turkish flies. In the UK and German populations, no acceptable concentrations of alkenes  
228 were detected. Only the Turkish population had a substantial concentration of alkenes in their  
229 chemical profiles.

230 To visualise the findings and to statistically differentiate the populations, PCA computations  
231 were carried out with the CHC data presented in Table 1. PCA was performed using six  
232 principal components, describing 95.7% of the dataset variation within the data set with the first  
233 three principal components, comprising 86.1%, 7.0% and 2.6%, respectively. Each sample was  
234 represented by a single data point in the graph, and each population had ten replicates. The 3D  
235 plot has been chosen because it allows for a more visual representation of the distinction

236 between the three different populations. In order to create the PCA plot seen in Figure 4, PC2,  
237 PC3, and PC4 scores were used.

238 Discriminant analysis was used to discover which CHCs were most significant for segregating  
239 populations of *C. vicina* adult flies from the UK, Germany, and Turkey. Compounds exhibiting  
240 large loading scores will be the most significant in the separation seen in the PCA plot. The  
241 main compounds with significant loading values in the PCA dataset were 4-Methylhexacosane,  
242 3-Methylheptacosane, 7-Methylheptacosane, and 3-Methyloctacosane compounds.  
243 Furthermore, since branched methyl alkanes had the highest total percentage among the CHC  
244 groups, it proved that these compounds were discriminant among the populations.

#### 245 **Empty puparia**

246 The chain lengths of the detected CHCs range from C21:H to C32:H. In the chemical profile of  
247 the UK population, 28 CHC compounds were detected, compared to 27 CHCs in the German  
248 population and 24 CHCs in the Turkish population (Table 2). The same number of n-alkanes  
249 was found in all three groups. The concentration of n-alkanes was much greater than that of the  
250 other CHC groups. Nevertheless, while the number of branched methyl alkanes was similar in  
251 the UK and German populations, fewer branched methyl alkanes were detected in the Turkish  
252 population. Only one alkene compound with sufficient concentration (>0.5%) was detected in  
253 the German population.

254 C27:H and C29:H (Table 2, compounds 8 and 22) were the most abundant n-alkanes in the  
255 populations of the UK and Turkey. While the C27:H in the UK population was the most highly  
256 concentrated than found in others, the C29:H concentration in the Turkish population was  
257 higher than in other populations. C25:H and C27:H (Table 2, compounds 4 and 8), on the other  
258 hand, were the most prevalent n-alkanes in the German population. The concentration of C25:H

259 found in the German population was significantly greater than those found in the populations  
260 of the UK and Turkey.

261 The dominant branched methyl alkanes were the same in all three populations, 11+13-MeC27,  
262 3-MeC27 and 11+13-MeC29 (Table 2, compounds 9, 13 and 24), but their concentrations varied  
263 between populations. While a branched methyl alkane could not be specifically detected for the  
264 Turkish population, 3-MeC21 and 5-MeC28 (Table 2, compounds 1 and 18) were detected only  
265 in the UK population, and 3-MeC30 and 3-MeC31 (Table 2, compounds 29 and 32) were  
266 detected only in the German population. Likewise, 13+15-MeC28 (Table 2, compound 15) was  
267 detected only in UK and German populations, and 11+13-MeC28 and 13+15-MeC29 (Table 2,  
268 compounds 16 and 23) were detected only in UK and Turkey populations. The presence or  
269 absence of branched methyl alkanes in the profiles or differences in their concentrations can be  
270 considered an indicator of discrimination between populations.

271 The chromatograms in Figure 5 represent the chemical profiles of *C. vicina* empty puparia  
272 collected from the UK, Germany and Turkey. Chromatograms were similar in general  
273 appearance. However, compound concentrations differed between populations. Whereas the  
274 CHC profiles of the populations of the UK and Germany were similar, the CHC concentrations  
275 in the Turkish population were different from them. The CHC concentrations detected in the  
276 Turkish population were higher than in the other two populations.

277 Figure 6 shows the total concentrations of CHC compounds detected in the CHC profiles of all  
278 three populations. According to the graph, the total n-alkane concentration detected in the  
279 Turkish population was 8% higher than in the UK, which had the lowest n-alkane concentration.  
280 On the other hand, the branched methyl alkane concentration detected in the UK population  
281 was 11% higher than the German population, with the lowest branched methyl alkane  
282 concentrations. Sufficient alkene concentrations (>0.5%) could not be detected in the UK and

283 Turkish populations. Only one alkene was detected at a 5% concentration in the German  
284 population.

285 PCA calculations were carried out using the CHC data presented in Table 2 to aid the  
286 visualisation of the findings and statistically discriminate between the different populations.  
287 PCA was performed using six principal components, describing 99.4% of the dataset variation  
288 within the data set with the first three principal components, comprising 85.4%, 12.8% and  
289 1.2%, respectively. The PC2, PC3, and PC4 components were used to create the PCA plot  
290 shown in Figure 7.

291 Discriminant analysis was used to discover which compounds were most significant for  
292 separating *C. vicina* empty puparia from the UK, Germany, and Turkey populations.  
293 Compounds exhibiting large loading scores will be the most significant in the separation seen  
294 in the PCA plot. The main compounds with significant loading values in the PCA dataset were  
295 pentacosane (C25:H), heptacosane (C27:H), and nonacosane (C29:H). As n-alkanes, especially  
296 OLAs, had the highest total percentage among the CHC groups, it showed that these n-alkanes  
297 were discriminant among the populations.

## 298 **Discussion**

299 The results showed qualitative and quantitative differences between the three populations of *C.*  
300 *vicina* in both adult flies and their empty puparia. These differences are also visible in the  
301 chromatograms showing the chemical profiles of the populations. The differences in compound  
302 concentrations across chromatograms suggested that CHC profiles of the same species are  
303 retained in a unique pattern and can also be used to distinguish populations of the same species  
304 living in different geographical areas. PCA analysis helps to visualise data from GC  
305 chromatograms that allow differentiating between populations. It was unnecessary to separate

306 the samples by sex for this research since the purpose was to investigate the variations in  
307 chemical profiles across various populations of the same species.

308 Moore et al. (2017a) determined the age-dependent chemical changes that occur within the  
309 adult profiles of *C. vicina*, *Calliphora vomitoria* (Linnaeus, 1758) and *Lucilia sericata* (Meigen,  
310 1826) over a period of 30 days, allowing for the age of the adult flies to be estimated. Therefore,  
311 to reduce the influence of age on the chemical profiles and examine the sole geographical effect,  
312 the adult fly populations used in this research were chosen as the first generation, derived from  
313 the *C. vicina* females collected from the wild and reared outdoors.

314 Howard and Blomquist (2005) reported that, in addition to being species-specific,  
315 geographically segregated populations might have qualitative and quantitative differences in  
316 CHC profiles depending on genetic factors and environmental conditions. Chapman et al.  
317 (1995) pointed out higher proportions of linear alkanes in populations living in environments  
318 with higher temperatures than other compounds. The general pattern is that species living in  
319 warmer and drier habitats are expected to lose water more rapidly than populations living in  
320 cooler and moister habitats. CHCs with longer carbon chains provide great potential against  
321 water loss (Gibbs, 1998b). Branched methyl alkanes prevent tight molecular packing, which  
322 reduces membrane fluidity and permeability (Drijfhout et al., 2010). Hot and dry ambient  
323 temperatures cause the loss of CHC with a short carbon chain that volatilises quickly. The  
324 presence of compounds with longer carbon chains in the CHC profiles of insects living in  
325 warmer climates supports the hypothesis that the epicuticular layer is regulated against water  
326 loss (Gibbs, 1998a, 1998b).

327 Studies focusing mainly on *Drosophila* spp. (Bontonou et al., 2013; Chung and Carroll, 2015;  
328 Ingleby et al., 2013; Markow and Toolson, 2013; Rajpurohit et al., 2017; Rouault et al., 2001,  
329 2004) have revealed differences among populations. However, few studies have been

330 conducted regarding its importance in forensic entomology; however, these studies were mostly  
331 carried out under controlled laboratory conditions. Byrne et al. (1995) examined the CHC  
332 profiles of both close and distant populations of *Phormia regina* (Meigen, 1826) adult blowflies  
333 collected from three locations. Populations were analysed using GC–MS, and identified the  
334 CHCs. Paula et al. (2017) determined the chemotaxonomic profile and intraspecific variation  
335 of CHC compounds in adults of the blowfly *Chrysomya megacephala* (Fabricius, 1794) using  
336 discriminant function analysis with GC-MS analysis. These studies successfully identified the  
337 species and distinguished them between the different locations. The results showed that there  
338 were qualitative and quantitative differences between samples from the populations, and the  
339 percentage of short carbon chain CHC was higher in the population living in a cooler and humid  
340 environment. In another study, Paula et al. (2020) demonstrated that mid-infrared photoacoustic  
341 spectroscopy could be used to evaluate the variation in chemical profiles of *C. megacephala*  
342 adults collected from different populations. The results obtained were supportive of the present  
343 study and supported that the chemical profiles of *C. vicina* adults vary between both close and  
344 more distant populations.

345 The usefulness of empty puparia in criminal investigations is tenuous, as they are difficult to  
346 identify depending on the species and are not narrowly defined in terms of age. They only show  
347 the completed development of at least the first colonisation wave. (Moore et al., 2022).  
348 However, they could be very useful in crimes where death occurred a long time ago, and empty  
349 puparia are the only entomological evidence available (Moore et al., 2017b). So far, only a few  
350 studies have investigated the identification of empty puparia, as well as how geographical  
351 location and the local temperature or environment could influence the chemical profiles of  
352 necrophagous flies and their puparia. Paula et al. (2018) developed a new method to determine  
353 the PMI based on chemical compounds of the empty puparia from different oviposition cycles  
354 of *C. megacephala*. They examined the chemical composition of 50 empty puparia from

355 different oviposition cycles using GC-MS. The results show that chemical profiles can be used  
356 to distinguish between empty puparia. Moore et al. (2022) analysed the chemical profiles of  
357 empty puparia from seven forensically important blowfly species. They used CHC profiles for  
358 identification and also investigated geographical differences by comparing profiles of the same  
359 species from different regions of four countries. Results showed differences between the  
360 profiles of *C. vicina* from Germany, Spain, Norway and England, and also geographical  
361 locations could be determined from this chemical analysis.

362 In the present study, the changes in the CHC composition of *C. vicina* adult flies and empty  
363 puparia between the UK, German, and Turkish populations could be explained by the variances  
364 in environmental circumstances, distance and climate. While the difference between the CHC  
365 profiles of the UK and Turkish populations was greater, the German population shared several  
366 characteristics with both of these populations. Between the CHC profiles of the UK and German  
367 populations, there was a larger degree of resemblance than between the CHC profiles of the  
368 German and Turkish populations. This might be attributed to the fact that the distance between  
369 the UK and Germany is relatively shorter and that the two countries have comparable climatic  
370 types. The Turkish population differed from the other two populations due to its geographical  
371 distance and different climate type. Species from humid climates like the UK have more alkenes  
372 and branched methyl alkanes than species living in drier habitats, such as in Turkey.

373 The changes in CHCs concentrations in the chromatograms of populations are primarily related  
374 to the complete chemical profile rather than specific compounds. PCA was applied to the  
375 dataset instead, as it would be rather difficult to distinguish the differences in the  
376 chromatograms visually. PCA has the advantage of not focusing on specific compounds, as it  
377 is not known in advance which compounds are the most indicative for identifying populations  
378 (Moore et al., 2013). However, there are a few peaks that stand out due to their significant PCA  
379 scores, indicating they are effective within the dataset. These values belonged to branched

380 methyl alkanes, the dominant group in adult flies, and n-alkanes, which were the most dominant  
381 compounds in empty puparia.

382 Limsopatham et al. (2018) examined the biological differences between the *C. vicina*  
383 populations in Germany and UK by comparing developmental rates, wing morphometrics, and  
384 molecular analyses. The separation of populations at a smaller geographic scale, however,  
385 remained unclear. Molecular phylogenetic analysis by the maximum likelihood method failed  
386 to distinguish between distinct geographic populations on a national or local level. DNA-based  
387 approaches may be a viable alternative to morphological analysis, as genotyping is often more  
388 rapid and straightforward. However, DNA analysis has several inherent risks, including DNA  
389 degradation, ineffective amplification, and contamination (Mazzanti et al., 2010).

390 The results present in this preliminary study revealed that the cuticular hydrocarbon analysis is  
391 a method capable of providing more information in forensic entomology. It could also serve as  
392 an indication of possible population differences beneficial in forensic casework. On a small  
393 scale, identifying variations between distinct populations of the same species at the national  
394 level will be the pioneer contribution to forensic entomology in determining the populations in  
395 different regions of the same country. Future studies that will include more species, locations  
396 and larger datasets are needed to understand the intraspecific variability better.

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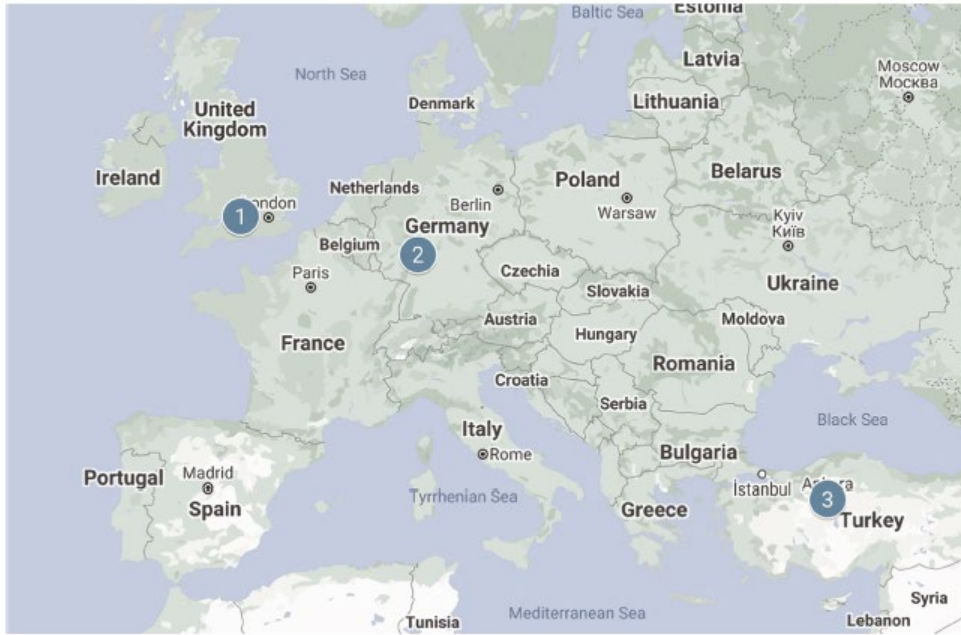


Fig. 1. Locations of collection sites for *C. vicina* populations.

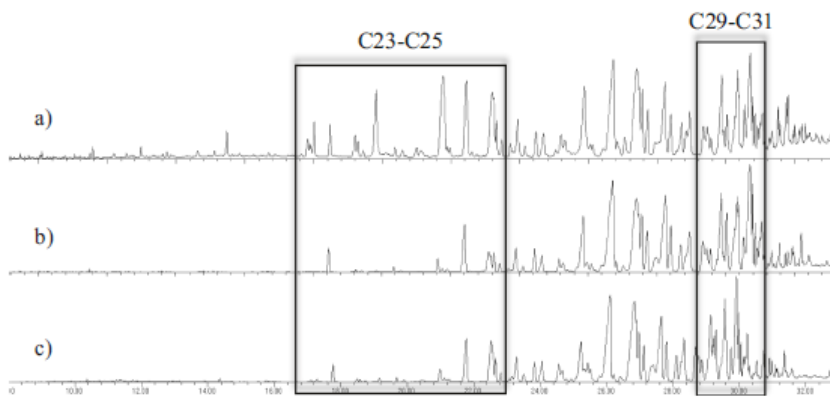


Fig. 2: GC chromatograms showing CHC profiles of *C. vicina* adult flies collected from a) Turkey, b) Germany, c) the United Kingdom, respectively. The marked areas show the population-specific key regions within the CHC profiles.

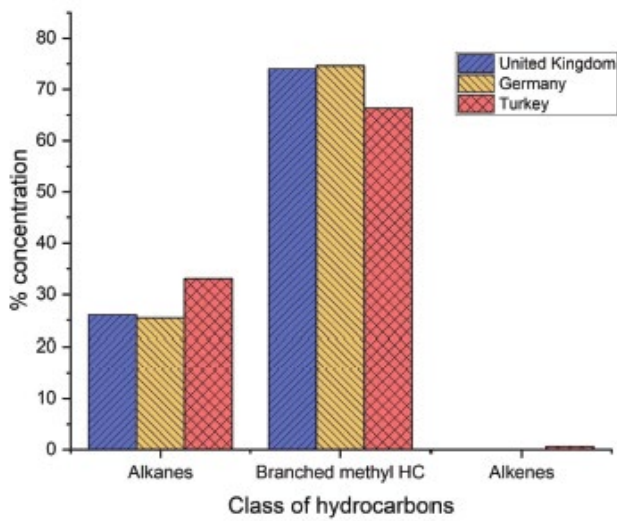


Fig. 3. Plot showing the total percentage concentrations of CHC classes detected in *C. vicina* adult flies.

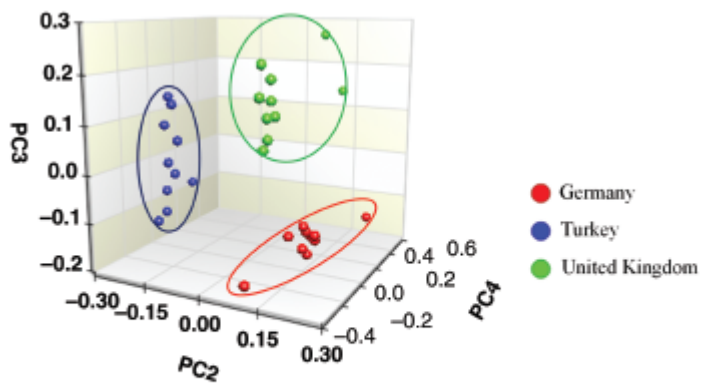


Fig. 4. PCA plot showing PC3 vs PC2 vs PC4 for *C. vicina* adult flies clusters, using n-alkanes, alkenes, and methyl branched alkanes. Clusters were grouped by population.

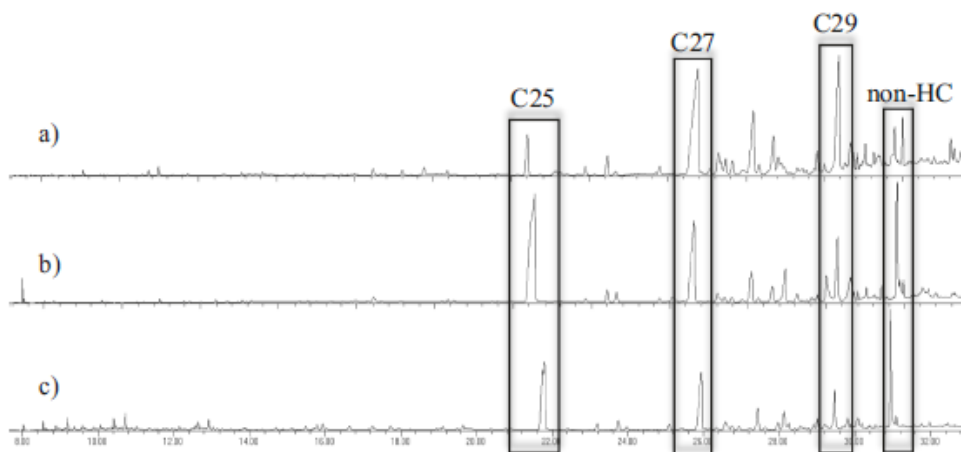


Fig. 5. GC chromatograms showing CHC profiles of *C. vicina* empty puparia collected from a) Turkey, b) Germany, c) the United Kingdom, respectively.

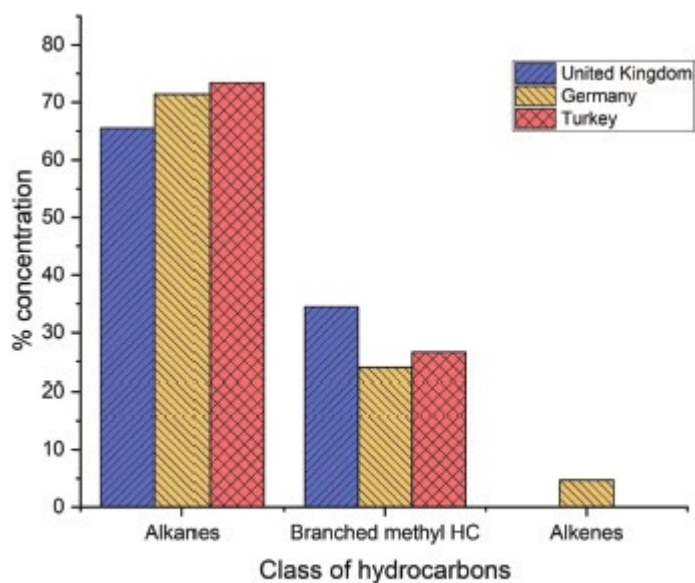


Fig. 6. Plot showing the total percentage concentrations of CHCs detected in *C. vicina* empty puparia.

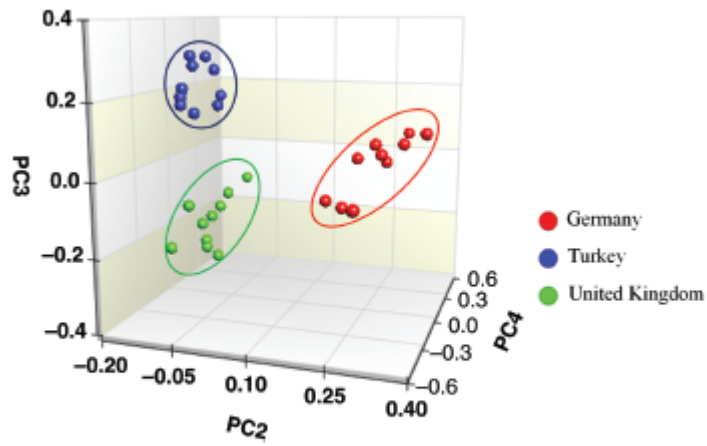


Fig. 7. PCA plot showing PC3 vs PC2 vs PC4 for *C. vicina* empty puparia clusters, using n-alkanes, alkenes, and methyl branched alkanes. Clusters were grouped by population.

**Table 1.** List of the compounds extracted from adult files of Turkish (TR), British (UK), and German (DE) populations of *C. vicina*, along with the total percentage peak area of each compound present and the percentage standard deviation

Peak number	Retention Time	Peak ID	<i>C. vicina</i> (UK)	<i>C. vicina</i> (DE)	<i>C. vicina</i> (TR)
1	17.779	C23	0.92 ± 0.34	0.68 ± 0.14	1.42 ± 1.05
2	18.587	7-MeC23	tr	tr	0.85 ± 0.62
3	19.693	C24	tr	tr	0.79 ± 0.59
4	21.041	3-MeC24	1.70 ± 1.15	ND	ND
5	21.802	C25	3.50 ± 1.58	2.81 ± 0.54	4.32 ± 1.65
6	22.555	11 + 13-MeC25	3.29 ± 1.24	1.57 ± 0.41	4.71 ± 2.97
7	21.151	9-MeC25	ND	ND	1.38 ± 0.40
8	22.691	7-MeC25	1.70 ± 2.09	0.68 ± 0.17	1.09 ± 0.26
9	23.079	5-MeC25	tr	tr	1.80 ± 1.05
10	23.163	4-MeC25	tr	tr	0.83 ± 0.50
11	23.314	3-MeC25	1.66 ± 0.64	1.28 ± 0.32	ND
12	23.846	C26	1.12 ± 0.47	1.33 ± 0.30	1.39 ± 0.31
13	24.087	13 + 15-MeC26	1.28 ± 0.33	1.24 ± 0.49	ND
14	24.583	11 + 13-MeC26	1.09 ± 0.23	0.91 ± 0.32	ND
15	24.690	8-MeC26	0.87 ± 0.57	0.58 ± 0.24	ND
16	23.876	5-MeC26	ND	3.18 ± 0.49	ND
17	25.296	4-MeC26	5.16 ± 3.13	0.64 ± 0.16	5.26 ± 3.13
18	25.813	3-MeC26	tr	0.71 ± 0.42	1.30 ± 1.31
19	24.169	Heptacosene	ND	ND	0.62 ± 0.18
20	26.145	C27	11.13 ± 6.11	10.99 ± 1.78	11.03 ± 2.76
21	26.440	13 + 15-MeC27	tr	0.72 ± 0.55	ND
22	26.890	11 + 13-MeC27	9.77 ± 3.10	9.94 ± 3.75	11.73 ± 2.54
23	27.024	7-MeC27	4.41 ± 7.72	2.59 ± 0.45	ND
24	27.375	6-MeC27	1.06 ± 0.99	ND	ND
25	27.421	5-MeC27	2.10 ± 0.96	2.47 ± 0.51	ND
26	27.682	4-MeC27	1.10 ± 0.76	2.32 ± 2.18	2.23 ± 0.89
27	27.844	3-MeC27	5.47 ± 1.89	6.22 ± 1.42	1.38 ± 0.59
28	28.138	C28	1.54 ± 0.73	2.07 ± 1.30	2.63 ± 0.76
29	28.363	13 + 15-MeC28	2.23 ± 0.67	3.62 ± 0.77	ND
30	28.708	11 + 13-MeC28	2.09 ± 0.94	2.10 ± 0.43	ND
31	28.799	8-MeC28	1.56 ± 1.03	1.52 ± 0.44	ND
32	28.886	6-MeC28	1.13 ± 0.62	1.50 ± 1.64	ND
33	29.171	4-MeC28	5.61 ± 3.96	4.90 ± 1.14	5.00 ± 1.57
34	29.305	3-MeC28	1.98 ± 0.95	3.06 ± 2.38	3.89 ± 2.65
35	29.590	C29	6.51 ± 3.42	6.20 ± 2.16	9.06 ± 2.56
36	29.763	13 + 15-MeC29	1.40 ± 0.73	2.31 ± 2.54	ND
37	29.940	11 + 13-MeC29	6.50 ± 3.25	7.71 ± 1.11	2.77 ± 0.87
38	30.001	7-MeC29	1.37 ± 0.24	1.95 ± 0.31	8.36 ± 2.45
39	30.134	5-MeC29	tr	0.58 ± 0.30	ND
40	30.185	4-MeC29	0.92 ± 0.50	0.82 ± 0.27	1.12 ± 0.40
41	30.251	3-MeC29	1.82 ± 0.90	2.19 ± 0.55	3.99 ± 1.91
42	30.527	C30	0.70 ± 0.28	tr	0.82 ± 0.21
43	30.736	8-MeC30	1.15 ± 0.69	1.11 ± 0.44	2.33 ± 0.83
44	30.910	4-MeC30	0.97 ± 0.69	1.10 ± 0.48	3.46 ± 0.98
45	30.985	3-MeC30	0.66 ± 0.21	tr	ND
46	31.106	C31	tr	0.82 ± 0.40	1.59 ± 0.72
47	31.142	13 + 15-MeC31	tr	0.65 ± 0.22	ND
48	29.993	11 + 13-MeC31	ND	0.56 ± 0.19	ND
49	31.360	9 + 11-MeC31	0.95 ± 0.43	1.61 ± 0.55	1.60 ± 0.64
50	31.590	3-MeC31	0.53 ± 0.20	1.20 ± 0.46	1.25 ± 0.37

nd: Not Detected.

tr: Detected in trace amounts (<0.5%).

**Table 2.** List of the compounds extracted from empty puparia of Turkish (TR), British (UK), and German (DE) populations of *C. vicina*, along with the total percentage peak area of each compound present and the percentage standard deviation

Peak Number	Retention Time	Peak ID	<i>C. vicina</i> (UK)	<i>C. vicina</i> (DE)	<i>C. vicina</i> (TR)
1	15.708	3-MeC21	1.44 ± 1.11	ND	ND
2	17.751	C23	tr	0.87 ± 0.49	0.83 ± 0.09
3	19.675	C24	tr	ND	0.55 ± 0.07
4	21.716	C25	4.52 ± 0.87	31.15 ± 6.66	4.96 ± 0.42
5	23.224	3-MeC25	0.57 ± 0.11	0.51 ± 0.21	0.73 ± 0.13
6	23.788	C26	3.74 ± 1.34	1.70 ± 0.50	2.23 ± 0.16
7	25.127	3-MeC26	0.93 ± 0.35	0.57 ± 0.17	0.90 ± 0.21
8	26.024	C27	36.66 ± 8.55	23.44 ± 5.36	32.02 ± 2.73
9	26.625	11 + 13-MeC27	4.19 ± 1.01	1.97 ± 0.76	3.04 ± 0.68
10	26.805	7-MeC27	1.31 ± 0.55	0.70 ± 0.25	1.21 ± 0.23
11	27.261	5-MeC27	1.13 ± 0.25	0.61 ± 0.20	0.98 ± 0.21
12	27.494	4-MeC27	0.99 ± 0.19	0.53 ± 0.17	0.79 ± 0.30
13	27.669	3-MeC27	8.13 ± 1.45	5.69 ± 1.68	7.50 ± 1.36
14	28.020	C28	4.71 ± 1.04	2.99 ± 0.96	5.99 ± 0.82
15	28.226	13 + 15-MeC28	1.21 ± 0.46	1.06 ± 0.20	ND
16	28.603	11 + 13-MeC28	1.37 ± 0.22	ND	0.62 ± 0.12
17	28.701	7-MeC28	0.58 ± 0.15	ND	0.57 ± 0.15
18	29.043	5-MeC28	1.74 ± 0.36	ND	ND
19	29.113	4-MeC28	0.32 ± 0.13	0.77 ± 0.25	1.68 ± 0.22
20	29.218	3-MeC28	2.24 ± 0.62	tr	0.90 ± 0.19
21	29.412	Nonacosene	ND	4.70 ± 1.21	ND
22	29.507	C29	14.10 ± 3.44	9.03 ± 2.26	21.34 ± 2.85
23	29.703	13 + 15-MeC29	0.72 ± 0.15	ND	1.27 ± 0.21
24	29.832	11 + 13-MeC29	3.92 ± 0.84	4.96 ± 1.15	3.06 ± 0.30
25	29.915	7-MeC29	1.18 ± 0.26	0.87 ± 0.28	0.94 ± 0.13
26	30.140	4-MeC29	0.83 ± 0.68	tr	ND
27	30.204	3-MeC29	1.64 ± 0.46	1.28 ± 0.37	2.38 ± 0.67
28	30.412	C30	0.36 ± 0.12	tr	1.66 ± 0.26
29	30.893	3-MeC30	ND	1.23 ± 3.08	ND
30	31.165	C31	0.96 ± 0.41	1.78 ± 0.59	3.82 ± 0.76
31	31.188	11 + 13-MeC31	ND	0.53 ± 0.17	ND
32	31.589	3-MeC31	ND	1.04 ± 0.85	ND
33	32.301	3-MeC32	ND	0.95 ± 0.38	ND

nd: Not Detected.

tr: Detected in trace amounts (<0.5%).

# Geographical variation of cuticular hydrocarbon profiles of adult flies and empty puparia amongst three populations of *Calliphora vicina* (Diptera: Calliphoridae)

Kula, Canan

2023-01

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