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

**Canan Kula[1,*], Jens Amendt[2], Falko P. Drijfhout[3] and Hannah E. Moore[1]**

[1]Cranfield Forensic Institute, Cranfield University, Defence Academy of the United Kingdom, Shrivenham, Wiltshire SN6 8LA, UK

[2]Institute of Legal Medicine, University Hospital Frankfurt/Main, Goethe-University, 60323 Frankfurt/Main, Germany

[3]Chemical Ecology Group, School of Chemical and Physical Science, Keele University, Keele ST5 5BG, UK

[*]Correspondence: c.kula@cranfield.ac.uk

## Abstract

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

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

*Keywords:*

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

## Introduction

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

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

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

For proving population-specific differences and habitat adaptations, which could be linked to a change in development, the differentiation or assignment of such clusters would be desirable. For this purpose, e.g. molecular biological and morphological analyses are suitable. (Kranz et 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 (alkanes 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.

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

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

## Materials and methods

### Insect materials

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

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

**Sample preparation**

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

**Chemical analysis of extracts**

Chemical analysis of extraction samples prepared in the laboratories of the relevant countries was performed in the same laboratory on the same Agilent Technologies 6890N Network GC system with a split/splitless injector at 250˚C, a Restek Rxi–1MS capillary column containing an SP of 100% Polydimethylsiloxane (30 m x 0.25 mmID, 0.25 µm 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

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

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

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

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

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

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

**Empty puparia**

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

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

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

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

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

Figure 6 shows the total concentrations of CHC compounds detected in the CHC profiles of all three populations. According to the graph, the total n-alkane concentration detected in the Turkish population was 8% higher than in the UK, which had the lowest n-alkane concentration. On the other hand, the branched methyl alkane concentration detected in the UK population was 11% higher than the German population, with the lowest branched methyl alkane 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

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

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

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

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

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

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

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

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

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