The temperature effect on the cuticular chemical profile of *Lucilia sericata* blowfly larvae

Canan Kula* and Hannah E. Moore

ABSTRACT

Forensic sciences, including forensic entomology, has seen rapid developments utilising new technologies. Cuticular hydrocarbon (CHC) analysis has become a useful tool for identifying and ageing forensically important blowfly species. CHC structure varies depending upon species, sex, age, and environmental conditions. Variation in hydrocarbon profiles may be significant for climatic adaptation, and protection against dehydration and desiccation. Existing literature shows differences in the hydrocarbon structures of wasps, beetles, fruit flies, and house flies when reared at different temperatures, but blowfly species have not yet been considered in such studies. Although many studies examined the relationship between temperature effect and developmental time of blowfly species using classical entomology methods, none have been conducted to determine whether the hydrocarbon structure of the blowfly species changes with temperature using CHC. In this respect, this study offers a pioneering contribution in the field of forensic entomology.

Keywords: forensic entomology, blowfly, *Lucilia sericata*, cuticular hydrocarbon, temperature effect

INTRODUCTION

Forensic entomology has become increasingly important in the last decade, and it is now well integrated in cases of homicide, suicide, or suspicious death, with insect specimens being taken into consideration as acceptable evidence alongside blood, fingerprinting, and other biological materials (Moore et al., 2014). When solving criminal cases, it is essential to determine the time since death, or post-mortem interval (PMI). There are various criteria and methods for the determination of the time of death in forensic medicine applications. These techniques use post-mortem changes, such as rigor mortis or livor mortis, related to fragmentation in traditional forensic pathology methods, for the time of death estimation. However, such estimates are limited to the first 72 hours after death (Campobasso, Di Vella and Introna, 2001). When human remains are found days, weeks, or even longer after death, conditions such as body temperature and rigor mortis or livor mortis are obsolete in estimating time since death. In these cases, insect life can play an essential role in determining the PMI (Amendt et al., 2011).

One of the crucial tasks of forensic science is to determine where and when the crime took place. Entomological evidence can provide valuable information about the estimation of the post-mortem interval and where the death occurred (Amendt et al., 2007). It is easy to determine whether a corpse has been removed from the real crime scene by comparing the geographical distribution of the species on the corpse and in the scene where the corpse is found (Catts and Goff, 1992). If the evidence shows that the place of death and where the corpse was found differ, a simulation carcass can be placed in the corpse discovery place to collect flies from that geographical area. Subsequently, the CHC structures of blowflies collected from animal carcasses and corpses are compared. A difference between the hydrocarbon profiles of the blowflies is an important proof of whether the corpse has been relocated after death, and indeed the differences in fly CHC profiles can provide evidence of the time of the relocation movement (Byrne et al., 1995). In this context, the effects on the hydrocarbon structure of environmental factors such as temperature, climate, and geographic location are becoming increasingly important in forensic investigations.

All terrestrial organisms undergo evaporative water loss, and insects and other arthropods are particularly susceptible to this due to their relatively small size. Maintaining water balance is crucial for the survival of insects, and water loss rates vary depending on temperature (Gibbs, 1998). Although there are multiple ways in which insects can manage water loss, the cuticle is the primary mechanism. A waterproof layer of straight-chain, methyl branched, and unsaturated

CHC mixtures with long chain lengths provides a hydrophobic barrier to water loss. If this layer is destroyed by physical methods, such as solvents or genetic approaches, the insect becomes very sensitive to desiccation. The ability of this CHC layer to prevent desiccation depends on its composition, which determines melting temperature. Longer-chain CHCs have higher melting temperatures (T_m), while methyl branched and unsaturated CHCs have lower melting temperatures. It is assumed that the composition of this waxy layer changes to manage water balance in order to adapt to new habitats or environments with different temperature and humidity values (Chung and Carroll, 2015).

Gibbs and Pomonis (1995) investigated the effects of chain length, methyl branching, and unsaturation on the physical properties of pure hydrocarbons by using Fourier transform infrared (FTIR) spectroscopy. It was found that T_m increases with chain length for n-alkanes, methyl alkanes, and alkenes, regardless of which end of the carbon backbone is extended. The results showed that methyl-branched alkanes have a lower melting temperature than n-alkanes. When the methyl branch is moved further into the internal position along the carbon backbone, T_m significantly decreases. Besides that, unsaturated hydrocarbons have a much lower T_m than their corresponding n-alkanes. Saturated and straight-chain hydrocarbon molecules melt at the highest temperatures, and T_m increases by 1 to 3ºC per carbon unit. The placement of a double bond, methyl branch or ester bond decreases T_m between 20 and 50ºC, depending on location. Compared to others, the chain length has relatively small effects on T_m.

Michelutti et al. (2018) researched the effect of temperatures on the cuticular chemical profile of social wasps. The results of the study showed that significant changes occur in cuticular compositions of the three wasp species compared to the control group. Quantitative and qualitative changes occurred in the chemical composition of both linear alkanes and branched alkanes and alkenes. Exposure to temperature changes demonstrably led to changes in the cuticular composition as a necessary mechanism for adaptation to new conditions. The studies show that there is a change in the hydrocarbon structure of wasps and beetle species reared at different temperatures. It was concluded that changing temperature conditions changes these species' hydrocarbon structure.

Since the non-polar structure of the hydrocarbons and the majority of the CHCs are volatile, chemical analysis of the extracts to determine hydrocarbons is carried out by gas chromatography (GC), a standard analysis method used since the 1960s. GC is a simple, fast, flexible, and relatively inexpensive method, and it is possible to perform in almost every

laboratory, with no requirements of long-term experience. Another essential feature of GC is its ability to separate up to 100 compounds in a single run. Detection of compounds separated by chromatography is performed by the detector. Commonly used standard detectors in GC are mass selective detector (MSD, or mass spectrometer) and flame ionization detector (FID) (Drijfhout, 2010).

In this study, the post-feeding larvae of *L. sericata* reared in the incubators at 14ºC, 25ºC, and 34ºC were used to investigate the effect of temperature changes on the cuticular hydrocarbon structure of *L. sericata*. The cuticular hydrocarbons were chemically extracted and analysed by using the mass spectrometry form of GC (GC-MS).

MATERIALS AND METHOD

The colony of *L. sericata* species belonging to the Calliphoridae family was collected from the wild in the UK (geographical origin: Shrivenham, Swindon, UK, 51°36'24.9"N 1°38'02.9"W) by trapping method for oviposition. The flies were supplied with blood, sugar, water, and dried skimmed milk powder. Fresh lamb liver was used as an oviposition medium, placed on a petri dish in a box with small perforations in the lid. Once eggs were laid on the oviposition medium, the petri dish containing the meat was transferred to and reared in incubators at 14ºC, 25ºC, and 34ºC. The cuticular hydrocarbons were extracted and analysed when larvae reached the post-feeding stage.

The liquid extraction method described by Moore (2013) was used for chemical extraction of hydrocarbons. For each specimen, 10 replicates (n = 10) were analysed to compensate for any changes in the extractions. For the extraction of hydrocarbons, larvae were placed into a 2 mL GC vial with hexane, ensuring that the insects were fully submerged and left for 10 to 15 minutes. Hexane, a non-polar solvent, was preferred as the hydrocarbons are non-polar components (Drijfhout, 2010; Moore, 2013). The hexane solution was transferred to column chromatography. Since the larvae were fed on meat, they were the only life stage needed to perform column chromatography. The column was made by plugging a Pasteur pipette with glass wool, followed by a small amount of silica gel. The larval extract from the GC vial was transferred to the column, and an additional 500 ml of hexane was added. The eluted hexane was collected into a clean GC vial and left until completely dry. The extracts were re-dissolved in 30 µl (for autosampler injections) of hexane, and a 2 ml aliquot was injected into the GC-MS. The effect of temperature changes on the cuticular structure was investigated using GC-MS.

RESULTS AND DISCUSSION

After rearing at temperatures of 14ºC, 25ºC, and 34ºC in constant temperature incubators, when the larvae reached the post-feeding stage, hydrocarbon profiles were successfully extracted and analysed by GC-MS, which detected 43 compounds, from which 24 hydrocarbon structures were identified (Table 1). The latter were found mainly to be a mixture of n-alkanes, methyl-branched alkanes, and alkenes. The n-alkanes range from C18: H to C34: H. Polar compounds and steroid compounds were excluded from the data set because the focus was on hydrocarbons, and they were less relevant to this study.

Table 1: Percent relative area of the compounds (> 0.1%) detected in the cuticle of *L. sericata* post-feeding larvae subjected to different temperature.

Time	**Compounds**	**14 °C**	**25 °C**	**34 °C**
		Percentage (% ± standard deviation)		
10.799	Octadecane	0.32±0.01	1.12±0.29	0.49±0.17
11.814	Nonadecane			0.45±0.08
12.232	Eicosene	1.05±0.89	1.58±0.21	0.53±0.06
13.033	Eicosane	1.17±0.19	2.24±1.11	1.36±0.48
13.956	3-Methyleicosane	0.95±0.20		
14.462	Heneicosane	1.38±0.21	2.47±1.02	1.33±0.40
14.779	2-Methylheneıcosane			1.05±0.33
16.095	Docosane	3.52±0.69	5.73±2.53	3.52±0.92
17.900	Tricosane	4.07±0.81	6.12±2.71	3.78±1.03
19.838	Tetracosane	3.83±0.74	5.75±2.62	3.64±1.11
21.916	Pentacosane	4.86±3.64	5.43±2.83	17.99±48.92
24.004	Hexacosane	1.90±0.47	3.44±1.48	1.49±0.57
26.075	Heptacosane	3.63±1.01	2.90±1.90	1.19±0.28
27.627	3-Methylheptacosane	1.65±0.42		
28.172	Octacosane	2.43±0.86	5.60±3.60	1.65±0.66
29.595	Nonacosane	13.59±4.24	18.59±12.39	15.12±7.66
30.289	13-Methylnonacosane	13.93±16.97		
30.485	6-Methylnonacosane	4.37±4.66	3.97±2.74	8.20±8.38
31.024	Hentriacontene	13.52±8.75		6.69±3.86
31.193	Hentriacontane	9.22±3.28	29.00±12.58	23.84±7.23
31.686	11-Methylhentricontane	5.07±4.10		
31.871	Dotriacontane			2.44±1.73
32.215	Tetratriacontadiene	6.89±3.71		
32.541	9-Methyltetratriacontane	2.67±2.30	6.05±2.49	5.25±3.19

The chromatogram in Figure 1 shows GC chromatogram retention times (between 13 and 30) of *L. sericata* larvae reared at three different temperatures (14ºC, 25ºC, and 34ºC). The results show that there is a massive increment in the percentage of each component of the larval hydrocarbon chains maintained at 35ºC. When the chromatograms are compared, it can be seen that while the larvae reared at 14ºC have more methyl branched alkanes, the larvae reared at 34ºC incubator have longer chain-length hydrocarbons than other larvae reared at cooler temperatures. Figure 2 displays a decrease in the number of methyl branched alkanes, and an increase in the number of long-chain molecules in the hydrocarbon structures. The viscosity and composition of hydrocarbons vary with temperature, because the water loss shows a positive correlation with the number of unsaturated bonds, and shows a negative association with the carbon-chain length (Gibbs, 1998).

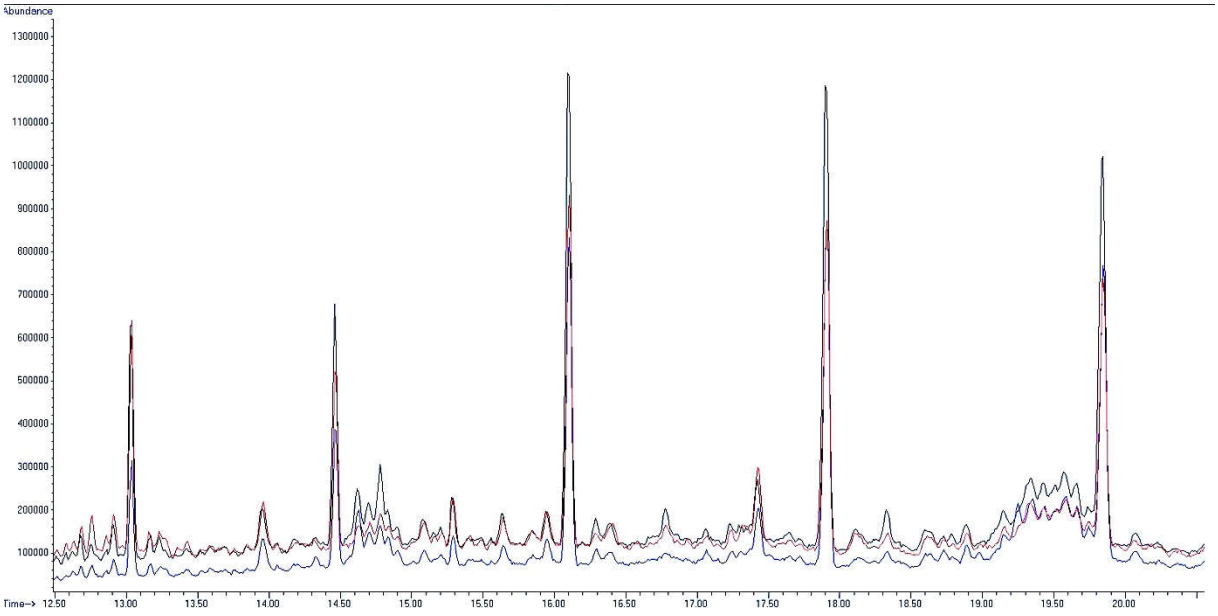

Figure 1: Zoomed GC chromatograms of *L. sericata* larvae reared at 14ºC, 25ºC, and 34ºC.

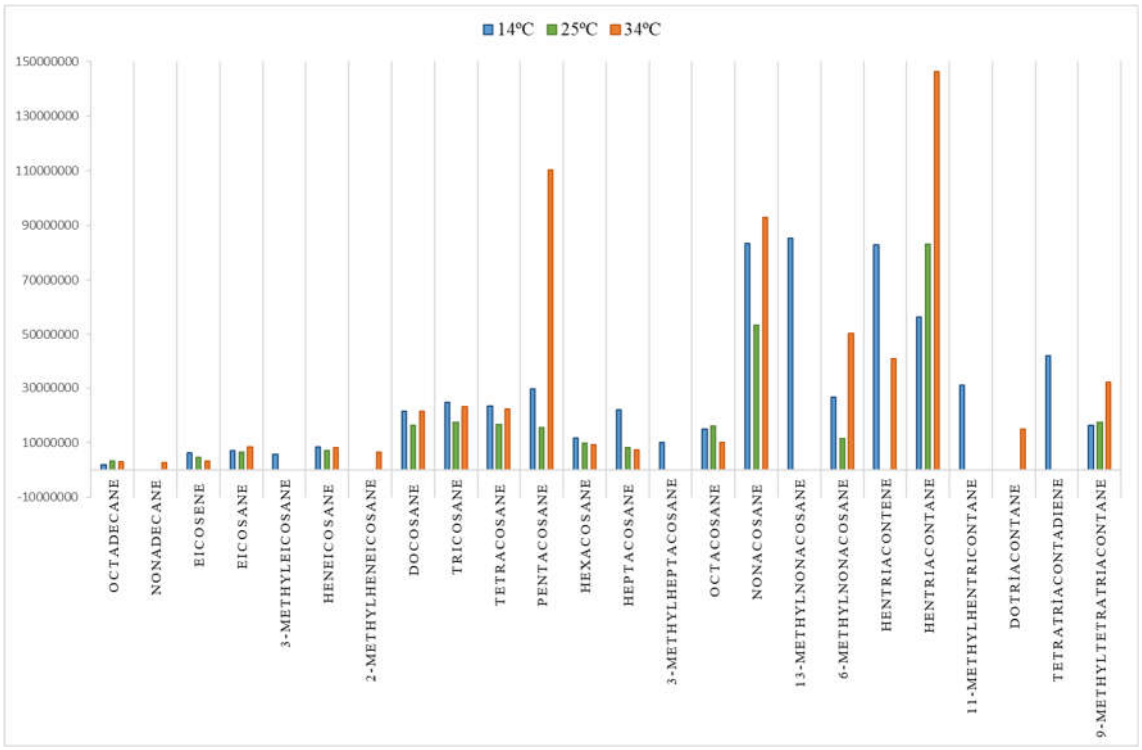

Figure 2: Graph of the average percentage peak area of the branched methyl alkane, alkene, and alkane compounds over the extraction period of *L. sericata* larvae reared at 14ºC, 25ºC, and 34ºC.

The proper deposition of additional hydrocarbons on the surface increases the hydrophobic nature of the surface and the diffusion path for water vapour, reducing water evaporation. The results showed that larvae kept at 34ºC had an increased number of long-chain length hydrocarbons, which have higher melting temperatures, which minimizes water loss and overcomes high-temperature effects. It was found that the larvae placed in a 14ºC incubator have a higher amount of methyl branched alkanes than those kept at warmer temperatures. This is due to the fact that larvae reared at lower environmental temperatures do not experience water loss problems. Long-chain length hydrocarbons are thought to provide superior protection against drying. These results are consistent with those of previous studies which found that higher water loss rate is associated with increased unsaturation and methyl branching in hydrocarbons (Gibbs and Pomonis, 1995; Gibbs, 1998; Hadley, 1977). Long-chain hydrocarbons tend to melt at higher temperatures, so higher melting temperature lipid structures lead to a reduction in the rate of water loss. Consequently, there was an increase in the number of long hydrocarbons in the cuticular structure of the larvae kept in a warmer environment, to prevent water evaporation and adapt to the environment.

It was concluded that changing temperature conditions change the hydrocarbon structure of species. Temperature plays a vital role in the production of cuticular hydrocarbons. This study indicates that increasing the number of carbon atoms in a hydrocarbon chain reduces the permeability of the membrane and larvae tend to adapt to relatively intense desiccation stresses as a result of high temperatures, while the presence of methyl branches has the opposite effect.

REFERENCES

Amendt, J., Campobasso, C.P., Gaudry, E., Reiter, C., LeBlanc, H.N., Hall, M. J. and European Association for Forensic Entomology (2007) 'Best practice in forensic entomology - Standards and guidelines', *International Journal of Legal Medicine*, 121(2), pp. 90–104.

Amendt, J., Richards, C.S., Campobasso, C.P., Zehner, R. and Hall, M.J. (2011) 'Forensic entomology: Applications and limitations', *Forensic Science, Medicine, and Pathology*, 7(4), pp. 379–392.

Byrne, A.L., Camann, M.A., Cyr, T.L., Catts, E.P. and Espelie, K.E. (1995) 'Forensic implications of biochemical differences among geographic populations of the black blow fly, *Phormia regina* (Meigen)', *Journal of Forensic Science*, 40(3), pp. 372–77.

Campobasso, C. Pietro, Di Vella, G. and Introna, F. (2001) 'Factors affecting decomposition and Diptera colonization', *Forensic Science International*, 120(1–2), pp. 18–27.

Catts, E. and Goff, M. (1992) 'Forensic entomology in criminal investigations', *Ann. Rev. Entomol*, 37, pp. 253–272.

Chung, H. and Carroll, S.B. (2015) 'Wax, sex and the origin of species: Dual roles of insect cuticular hydrocarbons in adaptation and mating', *BioEssays*, 37(7), pp. 822–830.

Drijfhout, F.P., J. Amendt, Campobasso, C.P., Goff, M.L. and Grassberger, M. (2010) 'Cuticular hydrocarbons: A new tool in forensic entomology?'. In: Amendt, J., Goff, M., Campobasso, C. and Grassberger, M. (Eds.) *Current concepts in forensic entomology*. Dortrecht, The Netherlands: Springer, pp. 179–204.

Gibbs, A. and Pomonis, J.G. (1995) 'Physical properties of insect cuticular hydrocarbons: The effects of chain length, methyl-branching and unsaturation', *Comparative Biochemistry and Physiology, Part B: Biochemistry and Molecular Biology*, 112(2), pp. 243–249.

Gibbs, A.G. (1998) 'Water-proofing properties of cuticular lipids', *American Zoologist*, 38(3), pp. 471–482.

Hadley, N.F. (1977) 'Epicuticular lipids of the desert tenebrionid beetle, *Eleodes armata*: seasonal and acclimatory effects on composition', *Insect Biochemistry*, 7(3), pp. 277–83.

Michelutti, K.B., Soares, E.R.P., Sguarizi-Antonio, D., Piva, R.C., Súarez, Y.R., Cardoso,

C.A.L. and Antonialli-Junior, W.F. (2018) ‘Influence of temperature on survival and cuticular chemical profile of social wasps’, *Journal of Thermal Biology*, 71, pp. 221–231.

Moore, H.E. (2013) Analysis of cuticular hydrocarbons in forensically important blowflies using mass spectrometry and its application in Post Mortem Interval estimations. PhD thesis, Keele University.

Moore, H.E., Adam, C.D. and Drijfhout, F.P. (2014) ‘Identifying 1st instar larvae for three forensically important blowfly species using “fingerprint” cuticular hydrocarbon analysis’, *Forensic Science International*, 240, pp. 48–53.

NAME-SURNAME : Canan Kula

NATIONALITY : Turkish

ADDRESS : Cranfield University

Defence Academy

Stephenson Lab 62/2

Swindon SN6 8LA

E-MAIL : c.kula@cranfield.ac.uk

Educations:

(11.2017 / …)	Defence and Security (PhD)
	Cranfield University – Defence Academy (UK)
(09.2016 / 08.2017)	Forensic Investigation (MSc.)
	Cranfield University – Defence Academy (UK)
(09.2008 / 03.2013)	Chemistry (BSc.)
	Hacettepe University (Turkey)