

CRANFIELD UNIVERSITY

CANAN KULA

CUTICULAR HYDROCARBON ANALYSIS FOR THE
IDENTIFICATION, AGEING AND GEOGRAPHICAL VARIATIONS
OF FORENSICALLY IMPORTANT BLOWFLIES

SCHOOL OF CRANFIELD DEFENCE AND SECURITY
PhD in Forensic Institute

PhD
Academic Year: 2017 - 2022

Supervisor: Dr Hannah E. Moore
Associate Supervisors: Dr Falko P. Drijfhout and
Professor Andrew Shortland

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Abstract

Calliphoridae (blowflies) are important for forensic entomology as they are often the first group of insects to inhabit decomposing remains. To estimate the minimum post-mortem interval (PMI_{min}), blowfly samples are collected from the crime scene and identified, and then the age needs to be determined for each species. Accurate species identification is crucial, as varied species have different life-stage timings. Previous studies on Diptera have shown the possibility of using cuticular hydrocarbon (CHC) analysis to aid species identification, ageing and ultimately calculating PMI_{min}. However, since prior research was conducted at constant temperatures, the effect of fluctuating temperature on chemical profiles and the stability of CHCs in the field remains unclear.

This thesis aims to examine the CHC profiles of *Lucilia sericata* and *Calliphora vicina* reared outdoors in the United Kingdom, Germany and Turkey in terms of species, age and inter-population differences. The first study investigates whether the stability of the CHC profiles of two colonies reared outdoors can be used to identify species-specific characteristics. The second examines the effects of fluctuating and constant temperatures on the CHC profiles of the life cycle stages and the use of their chemical profiles for age estimations. The final study aims to determine the differences in CHC profiles of the same species living in different habitats and whether they can be used to distinguish populations. The CHC profiles of the samples were extracted and analysed using Gas Chromatography-Mass Spectrometry (GC-MS). The statistical interpretation was carried out using Principal Component Analysis (PCA). The results showed that CHC profiles differed significantly between developmental stages by species, age, rearing temperature and geographical location. This study highlights that despite environmental factors affecting the stability of CHC compositions, the CHC analysis can be used as a complementary tool for identifying and ageing the species. Furthermore, the effects of ambient temperature and geographical variation on CHC profiles may be useful for determining inter-population variations.

Keywords: forensic science, Calliphoridae, blowfly, *L. sericata*, *C. vicina*, GC-MS, PCA

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To my lovely mother and father...

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List of Abbreviations

3D	Three-dimensional
ADD	Accumulated Degree Days
ADH	Accumulated Degree Hours
CHC	Cuticular Hydrocarbon
Cx:H	n-alkane
DA	Discrimination Analysis
DART-HRMS	Direct Analysis in Real Time High-Resolution Mass Spectrometry
DE	Germany
DNA	Deoxyribonucleic Acid
FID	Flame Ionisation Detector
GC-MS	Gas Chromatography - Mass Spectrometry
GLC	Gas-liquid Chromatography
[M ⁺]	Molecular Ion
<i>m/z</i>	Molecular weight/ion
mt DNA	Mitochondrial DNA
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PCs	Principal Components
PEI	Post-Ecdysis Interval
PIA	Period of Insect Activity
PMI	Post-Mortem Interval

PMI _{max}	Maximum Post-Mortem Interval
PMI _{min}	Minimum Post-Mortem Interval
PMSI	Post-Mortem Submergence Interval
SEM	Scanning Electron Microscopy
T _c	Critical Temperature
TLC	Thin-Layer Chromatography
T _m	Melting Temperature
TOC	Time of Colonization
TOF	Time of Flight
TR	Turkey
UK	United Kingdom
WLR	Water Loss Rates
xMeCy:H	Branched Methyl Alkane

Chapter 1

Introduction and Background

This chapter explains the background and structure of the thesis, which investigates the cuticular hydrocarbon profiles of two forensically important blowfly species, *Lucilia sericata* and *Calliphora vicina*. It is important first to assess the subject of forensic entomology. Therefore, this chapter gives a general overview of forensic entomology, including a brief history of the discipline, a brief introduction to the biology of blowflies and a discussion of the problems facing current methods and research in forensic entomology. It also exposes the description of the cuticle layer and the contributions of cuticular hydrocarbon analysis to forensic entomology. Finally, following the presentation of the existing literature, the research gaps identified and filled by the study, the aims and objectives of the thesis and the chapters that make up the thesis are discussed.

1.1 Forensic Entomology

Entomology is the general science of studying insects, while forensic entomology studies the application of necrophagous arthropods in criminal investigation [1]. In homicides, suicides and unexplained or suspicious deaths, insect samples such as Calliphoridae (blowflies) are considered as evidence, as is blood, fingerprints, hairs, fibres, or any other biological materials [2]. Entomological evidence can reveal important details such as when, where and how the death occurred, a possible pre-death or post-mortem medical history of the victim and geographical origin if a narcotic substance or drug has been identified [3,4]. Forensic entomology is divided into three categories: urban, stored-product and medicolegal. Urban forensic entomology examines cases and civil law

actions caused by termites, arthropods or other insect species that arise in environments where people live. Stored-product forensic entomology is concerned with insect invasion or contamination of a wide variety of commercial products. Medicolegal forensic entomology focuses on the role of arthropods in investigating crimes such as murder, suicide and rape. It also covers topics such as physical abuse and illegal trade [1]. Medicolegal forensic entomology will be the main focus due to the purpose of this thesis.

Medicolegal entomological techniques have allowed experts to gather reliable data for post-mortem estimations [5]. Forensic entomotoxicology is a part of medicolegal forensic entomology. Toxicological analysis of insects that fed on a corpse may indicate the presence of drugs if the victim was using them; this is particularly essential if the body has decomposed to an advanced stage as a tissue sample may not be available for analysis. Moreover, if illegal plant material such as cannabis is imported, any insect that can be detected on plants may be able to determine where the plant was cultivated. Medicolegal forensic entomology also covers cases of myiasis, which is an infestation of insects on a living person, a child or an elderly person. This condition suggests neglect or abuse, even if the infestation is discovered after the person's death [6].

The literature has often reported that the insect samples found on or near human cadavers are accepted as physical evidence and used as a legally valid procedure [7]. The first documented case of forensic entomology is 'The Washing Away of Wrongs', a medicolegal textbook written in the thirteenth century by the Chinese lawyer and death investigator Sung Tzu [8,9]. This book describes a stabbing near a rice field in China and the investigation that followed. After preliminary interrogations, the investigator gathered all the sickles of the villagers in front of the crowd. Although there was no apparent visual evidence on the sickles, adult flies swarmed to the invisible blood trace on one of the sickles. When confronted with this entomological evidence, the owner of the sickle confessed to the murder [10].

In the Middle Ages, the correlation between the larvae on a cadaver and the oviposition of adult flies could not be explained. However, by the seventeenth century, the metamorphosis of insects was more widely understood [11]. The first modern use of entomology in a criminal investigation was in a case in Paris where the corpse of a child was found concealed behind a plastered wall in a house. It was concluded from the remains of insects found around the corpse that the decomposition stage had occurred a

few years previously [12], in consequence of which the landlord was eliminated as being a murder suspect. Further theoretical advances were made by Megnin, who published a series of articles on medicolegal entomology between 1883 and 1898. The most famous of these, *La Faune des Cadavres* [13], popularised the concept that the medical and legal professions could find entomological data applicable to a wide range of forensic investigations (cited in [10]).

There are various criteria and methods for determining the time of death in forensic medicine. These techniques use post-mortem changes such as rigor/livor mortis, which are used in forensic pathology. However, their estimation can be limited to the first 72 hours after death [5,14]. When human remains are found days, weeks, months or even years after the death, body temperature and rigor/livor mortis can no longer be used to estimate the time of death. In these situations, insects play an essential role in estimating the minimum Post-Mortem Interval (PMI_{min}). PMI_{min} refers to the time between the death and discovery of a corpse [15]. The age of the oldest insect samples found on a corpse may provide evidence for PMI_{min} estimations [16]. When entomological data is used to estimate the time of death, there are many factors to consider. In the literature, the most recent studies mention both PMI_{min} and the period of insect activity (PIA) or time of colonisation (TOC) (Figure 1.1). In submerged corpses, post-mortem submergence interval (PMSI) has been used instead of PMI_{min} . The development time starts after the first female adult flies have laid their eggs, and the estimate of the PMI_{min} represents the most recent date or time at which death may occur. PMI_{min} refers to an event, rather than a period or interval and it provides more precise estimation when determining the duration of insect development. The time at which a person was last seen alive can be used to estimate the earliest possible date or time at which they may have died. This time is referred to as the maximum post-mortem interval (PMI_{max}) [17].

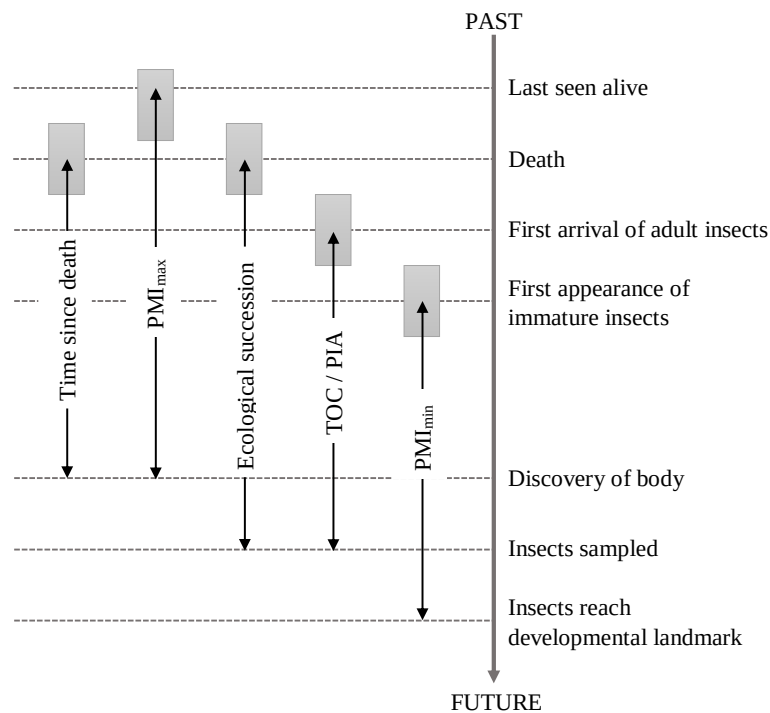


Figure 1.1: Relationships of the duration of insect development; minimum post-mortem interval (PMI_{min}), time of colonisation (TOC), a period of insect activity (PIA), maximum post-mortem interval (PMI_{max}) and events surrounding a death [17]

Entomological data can be used in two main methods to estimate PMI_{min} . The first is based on the time it takes for each species collected from the scene to develop and complete its life cycle and relates to corpses in the early stages of decomposition. In this regard, it is generally assumed that adult flies will discover the corpse soon after death, but this is not always the case, and such assumptions can lead to errors in the determination of PMI_{min} . Therefore, caution should be exercised when using this assumption, especially for indoor and closed container scenes or extreme weather conditions. The second fundamental principle concerns corpses in advanced stages of decomposition and is based on expected successive patterns of the arthropod population [1].

1.1.1 Stages of Decomposition

Decomposition is a natural process that begins at the time of death and continues until the skeleton is left after the soft tissues of the body have been broken down. Temperature, insect accessibility and activity, burial depth (if the body is buried) are the most important

environmental factors affecting corpse decomposition. Wounds and bruises that may be present on the corpse also increase the decomposition time of the corpse as open wounds make it easier for insects to reach soft tissue [1]. During the decomposition process, various insect species and other insects are observed on or around the corpse. As a result of previous research, it has been observed that depending on the ecological and biological tendencies of the insects, they are attracted by specific olfactory receptors and colonise the corpse at different stages of decomposition [15,16]. Some beetle species and their larvae are present both corpse and blowfly larvae that feed on the corpse. According to studies conducted by different researchers in different geographical regions, the decomposition period is described in five stages [6]:

1. Fresh: Morphological changes are minimal, and adult flies, specifically Calliphoridae, are drawn to the corpse to feed and lay eggs. This period lasts between 0-3 days, depending on the temperature, and it ends when the swelling begins.
2. Bloated: The body bloats and expands due to a build-up of gases produced by the cells and organs breaking down. There is a noticeable odour. Maggot masses may begin to form. The metabolic activity of feeding larvae might result in a localised rise in body temperature.
3. Decay: The skin is blackened and torn apart by the activity of Calliphoridae larvae. Thus, the odour increases and the decay continues. The beetles are also present at this stage, some feeding on the body and others preying on the larvae that feed on the tissues of the body. Early colonisers could be in the post-feeding stage and have started to migrate away from the body to find a suitable place for pupation.
4. Post-Decay: The majority of the soft tissues have been consumed, leaving just mummified skin, cartilage and bones on which beetles may feed.
5. Skeletal: Only bones and hair remain, with few or no insects present.

1.1.2 The Life Cycle of the Blowfly

Necrophagous insects immediately colonise a fresh corpse, given the accessibility and an amenable environment. They can detect an odour source shortly after death with higher

spatial precision. If fertilized female adult flies reach the corpse within a short time after death under favourable conditions, laying their eggs around natural orifices or wounds (if present) on the fresh corpse. The life cycle has six developmental stages from egg to adult fly (Figure 1.2). The larval stage includes the first, second and third instars. Larvae hatch from the egg and feed on soft tissues. When the third instar larvae finish feeding, the post-feeding stage begins. The post-feeding larvae leave the remains and begin to look for a dark and moist place to enter the pupation stage, whereupon they form puparia. It is the hardened layer of the third instar larvae. Adult flies emerge upon the completion of metamorphosis, and the life cycle begins again [15,18].

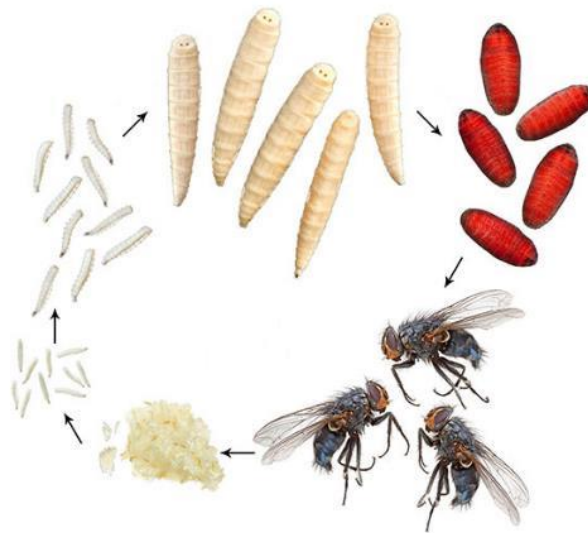


Figure 1.2: Blowfly life cycle [19]

Insects are important for forensic investigations as they can be used to estimate the PMI_{min} of a corpse [20]. It is known that Calliphoridae (Diptera) is usually the first group of insects that arrive upon and colonise fresh remains [21]; therefore, they usually form the primary forensic entomological evidence for estimating the time of death [22]. Calliphoridae is widely distributed in many parts of the world [23]. *Calliphora vomitoria* (Linnaeus, 1758), *Calliphora vicina* (Robineau-Desvoidy, 1830) and *Lucilia sericata* (Meigen, 1826) are the three most forensically important species and are among the first wave of necrophagous insects to colonise on decaying remains [7,24]. They are always followed by a sequence (or succession) of other adult flies and beetle species [25].

The time it takes for adult flies to hatch from eggs and complete metamorphosis typically varies between 2-4 weeks, depending on temperature and species. Various published developmental data provide information on the time taken for insects to develop through the life cycle at different temperatures, such as the growth rate of the larvae, developmental stages, the onset of the puparial stage and the hatching of the adult fly [26]. The development rate of insects depends primarily on temperature and food source and can vary even among closely related species [27].

1.1.3 Succession Pattern

A forensic entomologist must analyse the succession or development patterns of insects collected from crime scenes and corpses to estimate the PMI_{min} . The pre-appearance and presence intervals of some insect species can be determined regarding reference succession pattern studies. Such data are critical when using insects that colonise in the advanced stage of decomposition to obtain significant PMI_{min} [28]. The succession pattern also gives information about the geographical location, climatic conditions, the season of the year, habitat type, the physical state of the remains and the decomposition environment [29,30]. Therefore, the succession data collected in one region must be specific to the region [31]. A useful model should include data on the duration of activity of necrophile arthropods on carrion.

1.1.4 Species Identification in Forensic Entomology

If insect species are misidentified, this can cause a false estimation of the PMI_{min} because, at the same fixed temperature, different species will develop at different rates. Therefore, to be able to use the correct development data, accurate identification of necrophagous insect species is essential [32]. Even if local fauna is well known, identifying the species may not be easy, especially with immature stage larvae [1]. Prior studies conducted by Erzincioğlu [33], Liu and Greenberg [34], Amendt [2,15,35] and Szpila [36–38] provided the basic building blocks of species identification using morphological identification techniques. Drijfhout [39] and Moore [24,32,40–45] continue to make significant contributions to this field by using cuticular hydrocarbon (CHC) analysis.

1.1.5 Age Estimation in Forensic Entomology

A forensic entomologist has to accurately estimate the age of the oldest insect sample found on or around a corpse to estimate the time of death and the PMI_{min} . Identifying the species of insect samples is the first vital step in PMI_{min} determination, while the second is the age estimation of the identified species [16]. Each species has a different development rate, and within-species development can vary due to the impacts of crime scene-specific temperature and location conditions, as mentioned previously. Although immature larval stages are the most common entomological evidence encountered in early-stage decay, their age estimation is quite difficult because they are relatively small [42]. There are several methods for larval age estimation. Generally, morphological characters such as posterior spiracles are used to determine the instar stage of the larvae. Figure 1.3 shows the third instar posterior spiracles of *C. vicina* larvae.

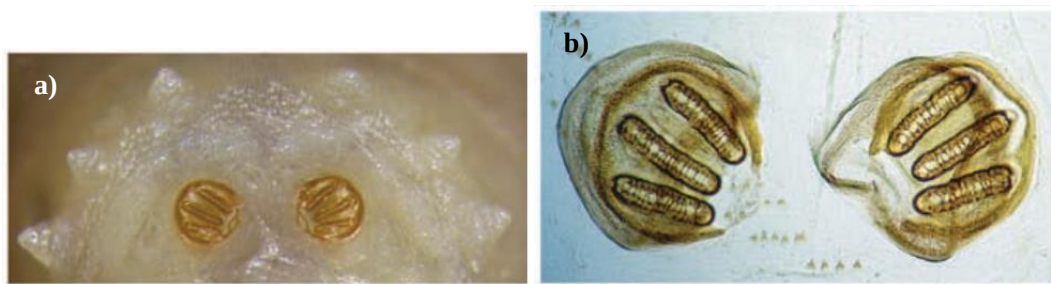


Figure 1.3: Third instar larvae of *C. vicina* (a) anal division, the upper half of spiracular field and (b) posterior spiracles [46]

Although traditional ageing methods are based on development rate and larval size, recent research has shown that analytical chemistry techniques have great potential in this area. For example, Moore [24,42–44] explained how species identification and age estimation could be undertaken more accurately with improved analytical techniques.

1.1.6 Current Methods in Forensic Entomology

Forensic entomology can provide valuable information by using recently developed techniques in a crime investigation, responding to questions about time of death, submersion interval, time of decapitation or dismemberment, relocation or storage of

corpse, identification of specific sites of trauma, drug use (entomotoxicology), linking to a suspicious crime scene, sexual harassment and identification of suspects [15].

There are two standard methods to estimate PMI_{min} . The first method compares the measured larval length on species-specific isomorphen and isomegalen diagrams, depending on temperature and time concerning larval size [47]. Secondly, the more commonly adopted approach calculates the accumulated degree hours or days (ADH/D) required for larvae to reach a specific developmental stage. Both methods provide an estimate of age and the PMI_{min} [48].

1.1.6.1 Isomorphen and Isomegalen Diagrams

Evidence to be used for entomological analysis found on and around the corpse should be collected and preserved by using the medicolegal standard procedures. The temperature of the scene where the larvae and pupae were collected should be determined instantly and accurately; then, the results should be compared with the weather temperature records [47]. Almost all methods used to age larvae are based on the knowledge that the development rate depends on temperature. Measurement of the length and weight of larvae is accepted as an indicator of age by entomologists; this information can be combined with temperature data to estimate the immature larval age and hence the PMI_{min} [49].

Isomorphen and isomegalen diagrams are usually used for developmental models. For the ageing of immature insects and estimating PMI_{min} , developmental models use measured size and developmental stage (e.g., first larval moult/ecdysis or pupariation) [15]. The growth rate is simply data on the developmental time of the immature stages recorded at different temperatures. However, the temperature used for research is usually constant but sometimes fluctuating.

The isomorphen diagram is a simple scatterplot that models the duration of developmental stages (from adult fly egg hatching to adult fly eclosion; X-axis) against temperature (Y-axis) (Figure 1.4). In this model, each contour represents one of the developmental stages. It is possible to accurately estimate PMI_{min} by knowing the developmental stage of the oldest immature insects in the corpse and the average environmental temperature of the crime scene. The isomorphen diagram only models the timing of developmental stages.

Generally, entomological evidence might not be collected live, and in some cases, live samples may be killed and preserved before analysis. The estimated age of insect specimens modelled from an isomorphen diagram will be less than their actual age since it has to be based on the last recorded developmental stage. In these cases, the more complex isomegalen diagram can be used to estimate PMI_{min} using dead larvae and fluctuating environmental temperature data [15].

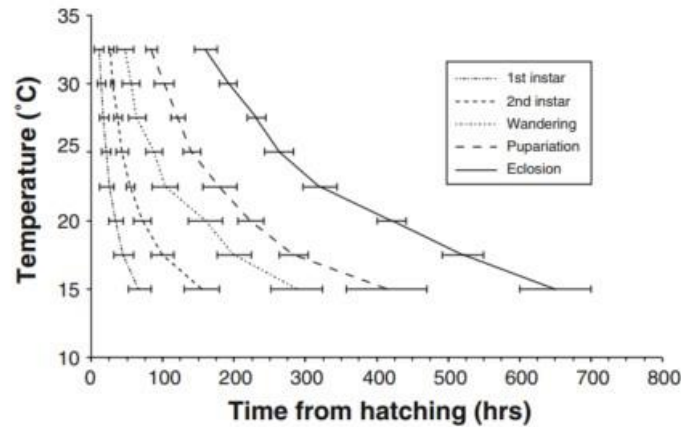


Figure 1.4: Isomorphen diagram showing life stages from oviposition to eclosion [15]

The growth curve data is used to generate the isomegalen diagram, a 3D contour plot that models larvae size (length, weight, or width) (Z-axis) as a measure of age versus temperature (Y-axis) and time (X-axis) (Figure 1.5) [15]. This high-resolution input provides a more accurate PMI_{min} estimation. Similar to the isomorphen diagram, the isomegalen diagram indicates all developmental data from oviposition to eclosion. The areas between the lines show the identical morphological stages of the species [47].

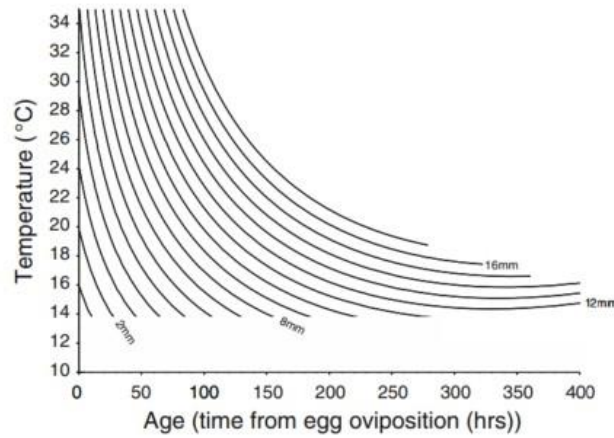


Figure 1.5: Isomegalen diagram of hypothetical development data [15]

Although these diagrams provide a more accurate estimation of PMI_{min} , insufficiencies remain in many ways. For instance, although the isomorphen diagram can be easily explained during legal proceedings due to its simplicity, this method can be used with live entomological evidence under known constant temperature conditions; however, stable and constant temperatures in real crime scenes are very rare [15]. The development rate from oviposition to eclosion may differ by geographical region. Even if the temperature is roughly constant in an indoor crime scene, the weather in the field fluctuates, so extreme care must be taken when interpreting the results [50]. In addition, larval size is not always an accurate measure of age, particularly for blowfly larvae. For example, it is known that the larvae size changes before entering the next developmental stage or before the pupariation or preservation process. In such cases, if age estimation is based on only the size of larvae, this may lead to incorrect PMI_{min} determination [51].

1.1.6.2 Accumulated Degree Hours or Days (ADH/D)

In the second method, the total amount of heat required for larvae or pupae to complete their developmental stage is called accumulated degree days (ADD) or accumulated degree hours (ADH). ADD/H is the product of time and temperature that accumulates between developmental thresholds for each day. This calculation is done as follows:

$$ADH = (Average\ temperature\ ^\circ C - threshold\ temperature\ ^\circ C) \times time\ (hours)$$

$$ADD = (Average\ temperature\ ^\circ C - threshold\ temperature\ ^\circ C) \times time\ (days)$$

ADD/H is the function of the temperature above the minimum growth threshold of a species and possibly geographic population and the time spent at that temperature. The temperature at which different species stop growing is the minimum developmental threshold temperature [23]. Some forensic entomologists use 0°C for all species, while others use 6-10°C. Reference studies [16,23,52,53] giving threshold values for individual species are used to calculate the ADD/H. Higher temperatures shorten the development time. Larvae can develop quicker and double their size at higher temperatures and mature within a few hours after hatching from eggs.

Ames and Turner [23] showed that the use of ADH values is questionable and may change according to climate, region, population and possibly even egg stock. They mentioned a criminal investigation in the southern UK where a skeletonised corpse was discovered in February. Some blowfly pupae evidence was found at the crime scene, and ADH was used to estimate PMI_{min}. The estimation, which covered quite long periods from November to February when the temperature was below 5°C, gave an erroneous result that was off by approximately a month. In this case, the ADH method underestimated the amount of development that occurred and thus prolonged PMI_{min}. According to ADH calculations, while the time of death was estimated to be mid-September of the previous year, the victim was actually still alive in mid-October. Therefore, using ADD/H in PMI_{min} estimation does not always give accurate results, so researchers are seeking new methods that give more accurate and reliable results.

In addition, the majority of information on the development of forensically important species has been gathered at consistent temperatures. Therefore, their thermal profiles resemble those of indoor environments more than those of outdoor habitats [54].

Investigators must collect the oldest larvae samples present at the crime scene. However, this is not always possible; sometimes, only pupae may be available. In this case, adult flies are expected to emerge, and species can be identified by morphological methods more easily [15]. However, such techniques assume that pupae are still alive and that the extra time required to complete pupae development can be taken for granted. Clearly damaged or dead pupae cannot be used for these methods; thus, forensic entomology experts have developed new chemical and DNA-based methods to complement morphological methods [55]. Many possible analysis methods have been proposed in

recent years. Among them, the most convenient method is based on analysing organic compounds extracted from the insect cuticle [52].

1.1.7 Modern Techniques in Forensic Entomology

Taxonomic descriptions are most easily and economically performed using categorical and qualitative morphological characters. However, if two species are closely related or different developmental stages of the same species are described, more quantitative characters may need to be used. Most identification methods use third instar larvae or adult flies because identifying the first instar larvae species is a complex procedure due to their relatively small size, requiring advanced taxonomic knowledge. However, many professionals who often collect entomological evidence in criminal cases have little training in taxonomy [56]. In some cases, larvae or pupae may die before reaching adulthood, so it may be impossible to identify. Ageing of species hence estimates the PMI_{min} correctly. In these cases, modern techniques gain importance [49].

Forensic entomology has benefitted from advances in molecular biology, and DNA technology has become a tool for forensic entomologists. Sperling et al. [57] were the first researchers to use the DNA-based species identification technique, and subsequently, different methods have been developed, such as examining the alloenzymes or mapping mitochondrial DNA by restriction enzyme digestions [58].

One of these techniques is the randomly amplified polymorphic DNA-polymerase chain reaction (PCR) technique, known as RAPD-PCR. This technique uses a single 10-nucleotide primer to amplify DNA regions surrounded by random primer sequences. It can use multiple DNA fragments that can be used as markers to identify individuals, populations, or species for genome mapping. Although RAPD-PCR is a highly sensitive method, it is difficult to replicate its results; before identification, hundreds of primers may need to be tested to distinguish between species or strains [59].

Another method that can be applied to all insect groups is restriction fragment length polymorphism-PCR (RFLP-PCR). The internally transcribed spacer (ITS) regions of the genome are expected to be highly changeable, but it is diagnostic for all species. In addition, ITS regions of ribosomal RNA gene repetitions have been recognized for the

diagnostic sequence characteristics of species, and this region is present in all organisms [59].

The mitochondrial genome is one of the methods used to identify different adult fly species. The use of PCR and sequencing techniques applied to an insect mitochondrial DNA (mt DNA) region allows species to be identified even in the case of extensive degradation. This is because the variation in mt DNA is enough to provide differences in sequences between close relative species [58].

DNA barcoding using the mitochondrial cytochrome oxidase gene is another developing method for species identification [60]. However, since it requires the development of comprehensive DNA barcodes for all species, it is a challenging method that still needs to be developed. Meng et al. [61] assembled the genome of forensically important species, *Aldrichina grahami* (Aldrich, 1930) (Diptera : Calliphoridae), at the chromosome-level using the Pacific BioSciences sequencing platform and the chromosomal conformation capture (Hi-C) technique. In another study, Liu et al. [62] examined eggs, first, second and third instar *A. grahami* larvae at three different temperatures, 4°C, 12°C and 20°C and performed RNA-sequence analysis. This study identified functional genes and demonstrated that various temperatures led to differences in the expression pattern.

An alternative is to extract the DNA from the pupae and identify species by sequencing a mitochondrial DNA locus [57,63,64]. Boehme et al. [20] studied the age estimation of *C. vicina* pupae by differential gene expression in metamorphosis. Examination by real-time PCR revealed that these transcripts vary significantly in temperature-dependent and age-specific characteristics. Davies and Harvey [65] studied the internal characteristics to produce a preservation and histology protocol suitable for routine forensic applications for blowfly pupae valuable for predicting potential age and PMI_{min} based on internal morphology. This study demonstrated that internal analysis using histology is possible, but they stated that its accuracy in age estimation is not known yet. In addition, they noted that many sections from multiple planes were required to get a complete picture of the internal pupae anatomy. Thus, multiple samples from the same age group were needed from the crime scene. There are notable morphological changes within the intra-puparia period that can estimate the insect age during metamorphosis. 3D micro-computed tomography (CT) can be used to view these changes [66].

The DNA-based analysis provides an advantage by introducing a new breadth to species identification and ageing methods. However, several disadvantages can limit the use of these techniques. DNA-based techniques are valuable, but they are time-consuming, expensive and destructive to the insect sample. Methods such as gene expression require deep molecular biology experience and extensive equipment to perform gene sequencing. In addition, as with any biological sample, pupae mt DNA has been shown to degrade with the age of the sample, and DNA degradation can profoundly affect amplicons [67].

With scientific and technological advances, scanning electron microscopy (SEM) [36], real-time high-resolution mass spectrometry [68], fluorescence analysis [26], histological techniques [65], and 3D micro-computed tomography (CT) [69] have also begun to be used in forensic entomology. Moreover, the combination of molecular and morphological methods can provide a more efficient strategy for estimating PMI_{min}.

Advanced imaging techniques such as SEM are used alongside DNA-based techniques for species identification. For example, Greenberg and Singh [70] examined the identification characters of 11 forensically important Calliphoridae eggs belonging to six species, including *C. vicina* and *L. sericata*. Using SEM, they identified the variability of morphological characteristics of eggs within and between populations of the same species, similarities between some congeneric species and similarities between some species from different species. Siri wattanarungsee et al. [71] studied the morphological characters of *Musca domestica* L. and *Chrysomya megacephala* (Fabricius, 1794) blowfly pupae using SEM. In order to identify the puparia, they used the size, body appearance and the number of papillae on the anterior spiracle, as well as the number of globules on the bubble membrane. However, advanced experience is required to identify these morphological characters. Furthermore, SEM is not found in every laboratory. Therefore, it is very rare to use SEM for species identification.

Amorim and Ribeiro [72] investigated the possibility of identifying pupae of *Ch. megacephala*, *Chrysomya putoria* (Wiedemann, 1818) and *Cochliomyia macellaria* (Fabricius, 1775) using a binocular microscope to evaluate inter-species differences in the posterior spiracle region. Unfortunately, they discovered only three valid characteristics for morphological identification: circum-spiraculum, whether it is obvious or not, the postspiraculum diameter and the lubricity of the empty puparia. Giffen et al. [68] analysed the chemical profile of the Calliphoridae family eggs: *C. vicina*, *L. sericata*,

Lucilia coeruleiviridis (Macquart, 1855), *Phormia regina* (Meigen, 1826) and members of Phoridae and Sarcophagidae families using direct analysis in real-time high-resolution mass spectrometry (DART-HRMS) and discrimination statistics. They showed that egg species could be grouped according to their chemical profile and mass spectrum. Although DART-HRMS can be used to distinguish between species, the applicability of this technique is lower because it is a rare device in forensic investigation laboratories.

Sukontason et al. [73] developed a light microscopy technique to identify eggs of the forensically important *Chrysomya rufifacies* (Macquart), *Ch. megacephala*, *Chrysomya pacifica* (Kurahashi), *Chrysomya nigripes* (Aubertin), *A. grahmi*, *Lucilia cuprina* (Wiedemann), *M. domestica* and *Megaselia scalaris* (Loew) species. They dyed the egg surfaces using a 1% potassium permanganate solution and viewed them under a light microscope. They used the plastron width, the morphology of the plastron area surrounding the micropyle and chorionic sculpturing, with the length of the egg as morphological separation characteristics [68]. However, this method failed to distinguish closely related species. In this method, discrimination is based on the existence of entomological keys to which images can be compared are required, and this is not available for all species yet. Recent studies suggest that hydrocarbon analysis on the epicuticular layer of insects can be a promising tool to identify an unknown sample or verify species identification in the case of uncertainty and provide an accurate age estimation [32].

1.2 Cuticular hydrocarbons (CHC)

The insects are coated with a lipid layer called a cuticle (Figure 1.6). A single layer of epidermal cells produces the cuticle called the hypodermis. The hypodermis is covered with a very thin epicuticular hydrophobic wax layer [74]. The epicuticular layer is formed by free lipids, a class of compounds comprising hydrocarbons, alcohols, fatty acids, waxes, acyl glycerides, phospholipids and glycolipids [39]. The predominant group in this structure is hydrocarbons, whose removal by organic solvents causes a significant increase in the desiccation rate [74]. Cuticular hydrocarbons (CHC) are biologically very stable and found in all developmental stages of insects [75].

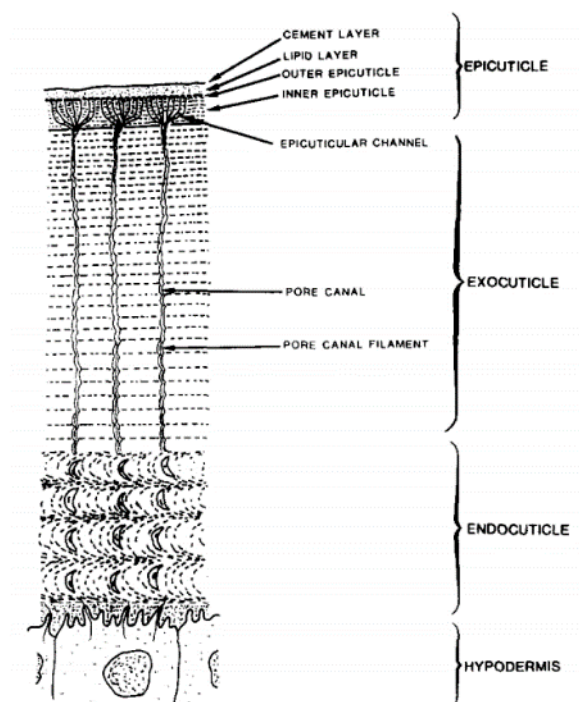


Figure 1.6: A section from the insect cuticle during endocuticle deposition [74]

Hydrocarbons are long linear molecules containing only carbon and hydrogen atoms. They are synthesized in specialised cells called oenocytes and are subsequently deposited in the insect cuticle. The cuticle was first identified as a waxy layer that controls water loss [76]. Hydrocarbons exist in saturated and unsaturated forms on insect cuticles and can have one or more methyl groups depending on the chain length (Figure 1.7) [39]. In saturated forms, such as n-alkanes or paraffin, all carbons are bonded with a single bond but may include one or more methyl groups. CHC profiles usually consist of n-alkanes and branched methyl alkanes. Linear alkanes are generally the group that dominates the CHC profile of insects. Unsaturated forms such as olefins may have one (alkene), two (alkadiene), or three (alkatriene) double bonds along the chain length. The olefins may be in the form of two isomeric structures termed cis-alkenes (Z-alkenes) or trans-alkenes (E-alkenes) [40].

All known insect cuticular alkenes have the ‘Z’ configuration. In addition, alkenes have two additional properties: the angle formed by the double bond; and the lengths of the chains on both sides of the double bond. This means that a single alkene may have different functions depending on the position of the double bond. For instance, the sex

pheromone of *M. domestica* adult flies is (Z9)-C23:1, with the double bond at the ninth position of the chain and (Z7)-C23:1 with the double bond at the seventh position [39].

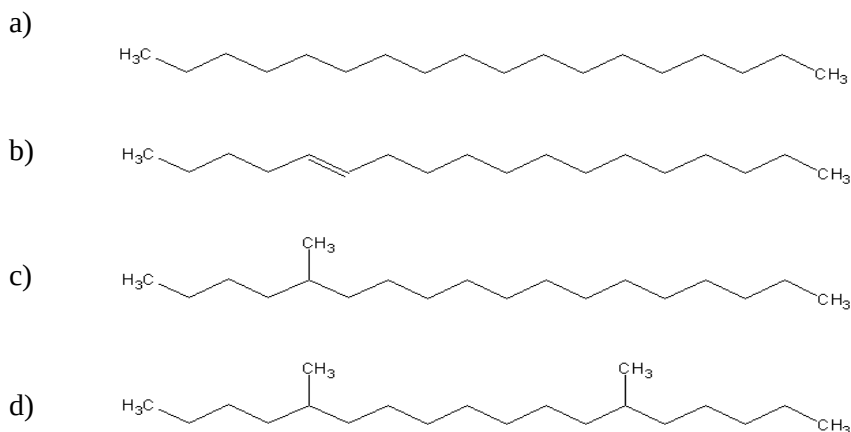


Figure 1.7: Linear long-chain cuticular hydrocarbons

a) Linear alkane: octadecane, b) Z-Alkene: (Z5)-octadecene, c) Branched methyl alkane: 5-methyloctadecane, d) Di-methyl alkane: 5,13-dimethyloctadecane

1.2.1 Cuticular Hydrocarbons in Entomology

Insect CHC profiles consist of more than 40 compounds whose composition varies depending on species, sex, sexual maturity and age [77]. Thus, there are many possible combinations making up an insect's chemical profile, with each insect species having its own unique, species-specific CHC profile [32]. In this context, using CHC compositions, it is possible to distinguish insect species, make age estimations and obtain information about their habitats; thus, determining PMI_{min} .

One of the essential tasks of forensic science is to determine where and when the crime occurred. Whether the corpse is transported from the actual crime scene can easily be understood by comparing the geographical distribution of the species on the corpse and in the scene where the corpse is found. However, it becomes difficult to understand if the insect found on the corpse is a common species [1]. CHC analysis is capable of providing a rapid and accurate comparison of CHC profiles across habitats, even at the larval stage.

Therefore, it is advantageous for the determination of the PMI_{min} and also where the death occurred in the criminal investigation.

CHCs are stable end products of genetically controlled metabolic pathways and are affected by factors such as reproductive status, stage of development, diet or temperature [39]. The function of the CHC profile is to prevent or reduce toxin effects (such as insecticides and bacterial and fungal pathogens) and function as pheromones or kairomones, which are essential for regulating adult insect behaviour (such as mate selection and ovulation) [78]. In addition, they prevent the evaporation of water through the cuticle and also function as communication signals. Chemical communication is critical for insects. CHCs facilitate communication by conveying various types of information, including for mate recognition, whereby CHCs are used like pheromones to attract potential mates. In addition, CHCs transmit information about nestmate recognition and function as fertility signals [76].

1.2.2 Cuticular Hydrocarbons in Forensic Entomology

1.2.2.1 Cuticular Hydrocarbons for Species Identification

CHC composition reflects the genotype and phenotype of an insect and is thus used in taxonomy [79]. CHC profiles usually contain 19-47 different compounds [80]. The presence, absence and relative amounts of the CHCs represent the surface chemical phenotype characteristics of these organisms [81]; thus, CHCs can be used to determine chemical characteristics for species identification. CHC analysis enables the identification of life stages that are more difficult to pinpoint using morphological criteria, such as eggs, immature larvae and empty puparia.

The use of CHCs for identification has been used for many years and is known as chemotaxonomy [82]. The CHC profiles vary quantitatively and qualitatively according to gender, population and development stage and are used for taxonomic separation [83]. This is explained in further detail in Chapter 4 (Species Identification Using Cuticular Hydrocarbons).

1.2.2.2 Cuticular Hydrocarbons for the Age Estimation

CHC analysis has the advantage of estimating the age to the day and complementing existing techniques used in forensic entomology [42]. Current studies have shown that the CHC composition in the larvae and pupae cuticle changes with age, and such changes are an integral part of larval development until eclosion [84]. Age estimation with CHC analysis is examined in detail in Chapter 5 (Age Estimation Using Cuticular Hydrocarbons).

1.2.2.3 Cuticular Hydrocarbons for Discriminating Geographical Populations

It is imperative to determine the place where the crime was committed in forensic investigations. Information about whether corpses have been transported can easily be determined if a species found on the corpse is not common in the area where the corpse was found. However, if only common species are detected on the corpse, it will not be possible to answer this question using morphological characters [85]. In this case, CHC analysis can play a critical role in determining the differences between populations.

Maintaining water balance is crucial for the survival of insects, including controlling water loss through the cuticle. A waterproof layer of straight-chain, branched methyl and unsaturated CHC mixtures with long-chain lengths provide a hydrophobic barrier. If this layer is destroyed by physical methods (e.g., solvents) or genetic methods, the insect becomes very sensitive to desiccation [74]. It is assumed that the composition of the epicuticular layer changes to manage water balance in order to adapt to new habitats or environments with different temperature and humidity values [76].

It is known that differences between populations depend on geographical distances that affect the degree of isolation and, in some cases, prevent gene flow between populations. Although the CHC profile tends to be specific, geographically separated populations may show qualitative or quantitative changes in CHC composition depending on environmental and genetic factors [85]. Specific CHC profiles are predominant in some taxa, and some have valid taxonomic characters. Even within the same species, the CHC composition may vary between populations [86,87]. Consequently, variations of CHC compounds can be used for taxonomy and biogeographical mapping. The geographical effect on CHC profiles of different populations is explained in detail in Chapter 6 (Comparative Studies of CHC Profiles of Egg, Larvae and Pupae for the UK and German

Populations) and Chapter 7 (Comparative Studies of CHC Profiles of Adult Flies and Empty Pupae for the UK and German and Turkish Populations).

1.2.3 Temperature Effect on Cuticular Hydrocarbon Profile

While the use of CHC analysis for species identification and age estimation of insects is getting increasing attention, the effect of environmental parameters such as temperature, climate, diet and laboratory rearing conditions on CHC production and stability has been overlooked [88]. Temperature is a variable that directly affects the distribution and concentration of organisms. Water loss rates (WLR) vary depending on temperature [86]. The waterproofing barrier in the epicuticle layer is characterized by weak molecular interactions, comparable in intensity to van der Waals interactions between alkane molecules. The physicochemical nature of the epicuticular layer is fundamental in determining the transcuticular water flux ratio. Therefore, the layer provides the adaptation of arthropods encountered in habitat desiccation stresses [89].

Beament [90] proposed a specific molecular orientation and packing for the surface lipids by the monolayer hypothesis based on the idea that surface lipids are amphipathic (the lipid molecules have both polar and non-polar regions). Under this monolayer, the polar ends of the lipid molecules interact with the cuticle, while the hydrophobic tails align themselves to maximize van der Waals interactions and minimize water flux. Above the “critical” or “transition” temperature (T_c), the tails change directions, so the water can pass and evaporate more easily. Gibbs [87] mentioned that while the rate of water loss in insects is relatively slow at lower temperatures, it increases rapidly above the T_c due to the difference in the permeability characteristics of the cuticle. Therefore, it is assumed that the surface lipids melt at T_c , whereby the epicuticular layer enters a fluid state; thus, water can easily be diffused.

The boiling points of the compounds in the insect cuticle must be higher enough to provide waterproofing at ambient temperatures and must be sufficiently fluid to be able to spread over the cuticle and be exchanged among nestmates. Among the hydrocarbon classes with the same number of carbons, n-alkanes have the highest boiling points, thereby increasing the hardness of the epicuticular layer. Furthermore, the boiling point increases in direct proportion to the carbon chain length. Extending an n-alkane by one CH_2 unit increases its boiling temperature by about 28°C [86]. Conversely, methyl groups

or double bonds distribute the crystal structure, lowering the boiling point. This depends on the location of the branched methyl group or double bond; however, the effect of chain length is usually much more significant [91].

Compared with branched methyl or unsaturated hydrocarbons, n-alkane molecules accumulate in a uniform structure due to van der Waals bonds, and this effect is even more significant at longer chain lengths. At lower temperatures, n-alkanes and monomethyl alkanes with strong van der Waals bonds can crystallize, which increases waterproofing and the diffusion of the substance and the viscosity of the epicuticular layer. This affects both the dehydration of the creature and the communication with the nestmates. On the other hand, methyl groups and unsaturation introduce disorder into the epicuticular layer, which reduces molecular aggregation [91]. The double bonds of alkene and alkadiene interfere with van der Waals interactions between molecules. This increases the fluidity and thus the permeability of the lipid layer [89].

There is a relationship between cuticle permeability and the physical properties of surface lipids [92]. In order to understand the functional physiological consequences of lipid variation, it is necessary to know the relationship between lipid structure and physical properties [93]. Increasing the number of carbon atoms in a hydrocarbon chain reduces the permeability of the cuticle, while methyl branches are assumed to have the opposite effect due to preventing the close packaging of hydrocarbon chains [94]. Gibbs [87] showed that South California populations of *Melanoplus sanguinipes* grasshopper species have chemical profiles that melt at higher temperatures than in northern populations. Southern populations lose less water at the same temperature, and warm-acclimated species have higher melting points than cold-acclimated ones [95]. Species in warmer and drier habitats lose less water than other temperate region species and have longer chain-length hydrocarbons.

Higher WLR is also associated with increases in unsaturation and methyl branching in hydrocarbons. Long-chain hydrocarbons tend to melt at higher temperatures, so the higher melting temperature (T_m) lipid structures reduce the rate of water loss [93]. The melting point affects the waterproofing ability of the epicuticular layer. If the ambient temperature is above the melting point, the water loss from the epicuticular layer is significantly increased. Hydrocarbon mixtures have a melting range rather than a melting point; thus, melting occurs continuously over a more comprehensive temperature range

and not just at a specific temperature [91]. The longer hydrocarbon molecules that influence membrane fluidity tend to reduce epicuticular permeability to water molecules, despite the effect of methyl branches on van der Waals interactions. Although alkadiene displays a similar effect on epicuticular permeability, this is not as clear as in branched methyl alkanes. Adding only two carbon atoms to a C25-alkadiene has remarkably potent impacts on cuticular permeability, indicating a kind of threshold effect [89]. Alkenes containing 30 or fewer carbons, found most often on insect cuticles, melt below 25°C, while n-alkanes generally melt above 45°C. For example, n-tricosane, with 23 carbons, melts at 47°C, while n-tritriacontane, with 33 carbons, melts at 70°C [93]. The viscosity and composition of hydrocarbons vary with temperature because the loss of water shows a positive correlation with the number of unsaturated bonds and shows a negative correlation with the carbon-chain length [86].

The cuticle provides signalling in interspecific and intraspecific interactions. CHC compounds can also be rearranged to provide communication [96]. Therefore, this communication task of CHC components is considered an explanation of branched methyl and alkene structures that cause dehydration and reduce the melting temperature of the structure at higher environmental temperatures [86,97]. Previous studies have argued that while the short-chain CHCs are considered to be predominantly involved in pheromonal communication, long-chain CHCs are believed to be predominantly involved in waterproofing [98]. Thus, linear alkanes are responsible for waterproofing and branched methyl alkanes and alkenes are responsible for chemical signalling or communication [97].

Gibbs and Pomonis [93] investigated the effects of chain length, methyl branching and unsaturation on the physical properties of pure hydrocarbons using Fourier transform infrared (FTIR) spectroscopy. It was found that the T_m increased with chain length for n-alkanes, methyl alkanes and alkenes regardless of which end of the carbon backbone was extended. The results showed that branched methyl alkanes have a lower T_m than n-alkanes, and when the methyl branch is moved further into the internal position along the carbon backbone, the T_m is significantly reduced. Furthermore, 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 1-3°C per carbon unit. The placement of a double bond, methyl branch or ester bond decreases T_m between 20 and 50 °C, depending on location. Compared to others, the chain length has

relatively minor effects on T_m . In addition, the surface lipids (branched, unsaturated or otherwise modified) used in communication generally reduce T_m [95]. Gibbs [95] described that phase separation occurs at ecologically relevant temperatures. In many insect cuticles, regions of highly permeable fused alkenes and poorly permeable, solid crystalline alkanes exist as discrete segments (Figure 1.8).

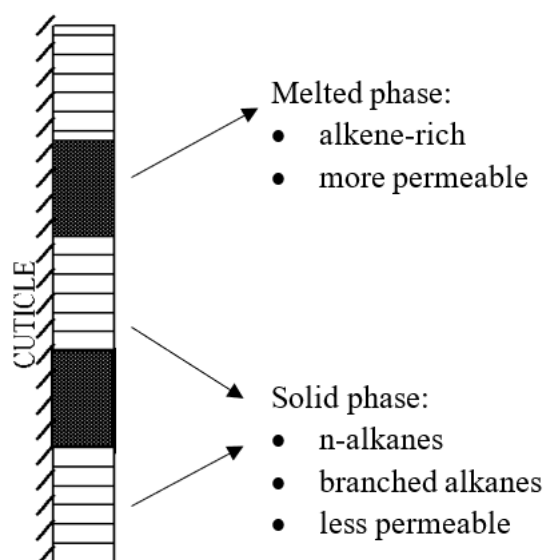


Figure 1.8: Hypothetical model for phase separation of cuticular lipids [95]

Hadley [94] collected *Eleodes armata* LeC. from desert habitats during the winter and summer seasons. The results showed that the winter active beetles increase the chemical profile to overcome the new higher temperature effects by reducing the WLR. Summer-active beetles have a higher proportion of branched methyl alkanes and a longer chain length than those collected in the winter. The longer chain length CHCs are thought to provide the summer-active beetles with more excellent protection from desiccation. This study proved that increasing the number of carbon atoms in a carbon chain reduces the permeability of the membrane, while the presence of methyl branches has the opposite effect.

Toolson [89] demonstrated the effects of different temperature regimes on epicuticular layer composition and cuticular permeability in *Drosophila pseudoobscura*. Adult flies hatching from pupae maintained at 24°C showed remarkably lower WLR than those hatching from pupae reared at 17°C. The components that tend to reduce the WLR are

directly proportional to the puparial rearing temperature, while the components increasing the WLR are inversely proportional to the puparial rearing temperature. Exposure to higher temperatures in the puparial stages synthesised the epicuticular layer, which adapted the adult flies to relatively intense desiccation stresses due to higher temperatures and lower relative humidity. According to the results, the WLR of adult flies with epicuticular layer extracted with hexane is incredibly high. If that could be applied to live adult flies, the adult flies would desiccate and die within a few minutes. The results show that the thermal regime has a significant effect on the WLR.

Savarit and Ferveur [75] analysed the CHC profiles of *Drosophila melanogaster* adult flies reared at constant temperatures and shifting temperatures, finding that increasing the temperature from 20°C to 25°C caused a decrease in the number of short-chain n-alkanes and increased the amount of the longer chain CHC in the chemical profiles. Furthermore, different populations were affected differently by the temperature increase. While the shorter carbon chain levels in one population decreased and longer carbon chains increased, in another population, all CHC levels decreased. These results are consistent with Gibbs's results, which suggest that species from warmer and drier habitats contain longer carbon chain CHCs than those from temperate climates [86]. Furthermore, both species maintained in the laboratory during at least several hundred generations have been found to maintain different genetic potentials by responding differently to temperature changes. Thus, this study supports that temperature has a relatively significant effect on the adaptation of CHC profiles.

Noorman and Otter [99] examined the effects of relative humidity, temperature and population density on chemical profile in male and female *M. domestica* adult flies. The results showed that while male adult flies at 35°C produced about 80% more CHCs than those reared at 20°C, female adult flies at 35°C produced about 50% more than those reared at 20°C. Although there was no significant difference in the relative amounts of the various CHCs produced at the two temperatures of the male adult flies, the relative amounts of nonacosane and methyl- and dimethylnonacosanes in the female adult flies were found to be higher at 35°C than at 20°C. The T_m of the mixture of all CHCs was 36.8°C in females and 39.4°C in male adult flies. The T_m of all CHCs is higher in males than in females [93]; thus, keeping the T_m of the cuticular lipid mixture within a certain range may affect the amounts at which certain hydrocarbons are produced, possibly due

to different environmental conditions. Therefore, there is no reason to vary the amount and composition of CHCs in male adult flies at ambient temperatures.

1.3 Aim and Objectives

The main aim of the research presented in this thesis is to examine cuticular hydrocarbon profiles of the two forensically important blowfly species, *Calliphora vicina* and *Lucilia sericata*, to test the following hypotheses:

1. The CHC profiles of the species reared in the field can be used for species identification.
2. The CHC profiles of the life stages of species, including eggs, larvae, pupae, adult flies and their empty puparia, vary depending on age and temperature, and this change can be used for age estimations.
3. Temperature fluctuations and stability have impacts on the CHC profiles of the development stages of the species.
4. Different populations of the same species could have interspecific variations on CHC profiles.

The following research objectives have been formulated to achieve this aim:

- To test the stability of CHC profiles of the species reared in the fields at ambient temperature.
- To investigate the age-related changes in the CHC profiles of the species reared outdoors.
- To examine the temperature effects on the CHC profiles of the developmental stages of species reared at ambient temperatures and a constant temperature in the laboratory.
- To evaluate and compare the interpopulation differences amongst the populations of the same species collected from Turkey, Germany and the United Kingdom and reared outdoors.
- The necessity of outdoor verification studies to take the CHC technique one step further.

1.4 Thesis Layout

This thesis consists of eight chapters and several sections, subsections and sub-subsections within each chapter. The following describes the structure and layout of this thesis.

Chapter 1: *Introduction and background* provide aims and objectives, thesis layout, an overview of the background, advances in the area of forensic entomology and the cuticular hydrocarbon method, as well as a review of relevant literature and prior studies.

Chapter 2: *Techniques and Data Analysis* comprehensively reviews the literature on the Gas Chromatography-Mass Spectrometer (GC-MS) and Principal Component Analysis (PCA) techniques utilised in the research.

Chapter 3: *Materials and Method* describe the research, the experimental method itself, chemical analysis and the materials used.

Chapter 4: *Species Identification Using Cuticular Hydrocarbons* covers the identification of species for the life cycle stages of *L. sericata* and *C. vicina* species reared outdoors, as well as the results obtained and the discussion that followed the findings.

Chapter 5: *Age Estimation Using Cuticular Hydrocarbons* examines the estimations of ages during developmental stages of the life cycles for two species reared outdoors, as well as the results and discussion.

Chapter 6: *Comparative Studies of CHC Profiles of Egg, Larvae and Pupae for UK and German Populations* comparatively examine egg, larval and puparial life stages of species from the UK and Germany.

Chapter 7: *Comparative Studies of CHC Profiles of Adult Flies and Empty Puparia for the UK and German and Turkish Populations* comparatively examines adult flies and their empty puparia of species from the UK, Germany and Turkey.

Chapter 8: *Summary, Conclusion and Future Research* summarizes the main findings of the studies presented in this thesis, including a conclusion of the experiments performed and offers suggestions for future research.

Chapter 2

Techniques and Data Analysis

2.1 Introduction

This chapter explains the theory and principles of analytical instruments and computational techniques used to analyse and interpret data. The primary analytical technique used in this thesis was Gas Chromatography-Mass Spectrometry (GC-MS). In addition to the combined GC-MS technique, GC and MS techniques are discussed separately and as the basic components of each instrument. The statistical method of PCA is also briefly explained. This technique was used to visualize variation in the dataset obtained from the GC chromatograms.

2.2 Chromatography

Chromatography is a popular method that separates and identifies the chemical components of complex or unknown mixtures [100]. Chromatography needs two stages to separate analytes from complex mixtures: stationary and mobile phases. Chromatography separates components in a sample mixture according to differences in migration rates by carrying compounds and mixtures through the stationary phase with a mobile phase flow. The sample components are known as solutes or analytes [101].

Chromatography methods are divided into two groups, column and planar chromatography. In column chromatography, the stationary phase is held in a narrow tube, and the mobile phase is forced to flow through the tube under pressure or gravity. In planar chromatography, the stationary phase is coated on a flat plate or a porous paper, and the mobile phase moves through the stationary phase by capillary action or gravity

[100]. There is a specific application area for each type of chromatography technique, depending on the sample type, analytes to be separated, the column technology used to separate analytes and the type of detection system [102].

2.3 Gas Chromatography (GC)

Column chromatography methods are divided into three groups (Figure 2.1): liquid, gas and supercritical fluid, according to the physical state of the mobile phase. For GC, the mobile stage is gas, whereas, in liquid chromatography (LC), the mobile phase is used in liquid [101]. GC is an analytical technique used to separate volatile (gas phase) organic compounds [102]. To perform separation by GC, the sample containing the different component mixtures is first vaporised. The mobile gas phase (carrier gas) is responsible for carrying the vaporised sample along the column. The samples are partitioned (equilibrated) into the stationary liquid phase in accordance with their solubility in the stationary phase at a given temperature. Depending on their relative vapour pressure and affinity for the stationary phase, the sample components are separated from one another [101]. The mobile phase is a constant flow and must not interact with the analyte molecules. The primary mission of the mobile phase is to transport the analyte through the column [100].

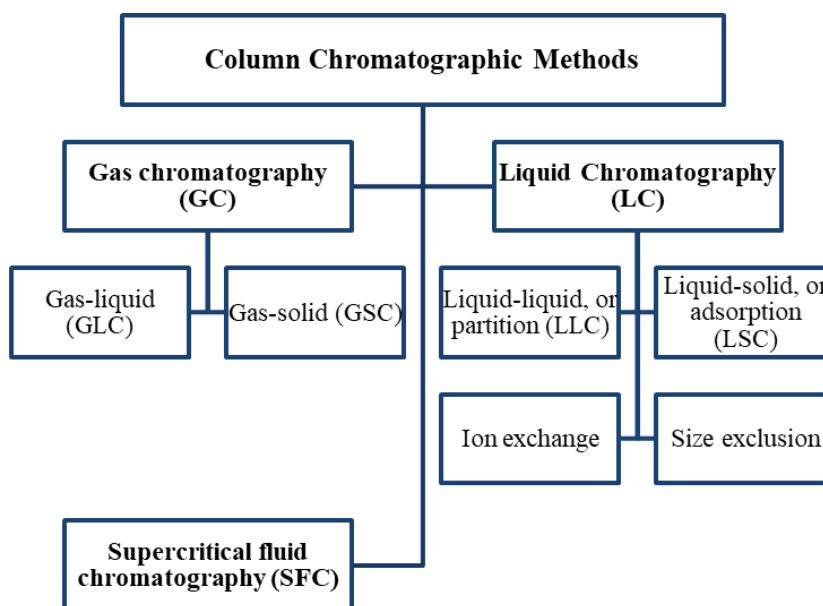


Figure 2.1: Classification of column chromatography methods according to the physical state of the mobile phase [100]

There are two types of gas chromatography based on the stationary phase type: gas-liquid chromatography (GLC) and gas-solid chromatography (GSC). In the GLC method, the analyte is separated between a gas mobile phase and a liquid stationary phase. GLC is commonly used synonymously with known GC, and it is the most preferred technique in all scientific fields. GLC is used to analyse highly volatile and thermally stable components at temperatures up to several hundred degrees Celsius. In gas-solid chromatography, the stationary phase is solid and allows analyte retention due to physical adsorption [101].

2.3.1 Chromatogram

A detector that responds to solute concentration is placed at the end of the column during elution. The detector plots the signal as a function of time or the volume of the mobile phase added, and a series of peaks is obtained, forming a plot known as a chromatogram. The positions of the peak areas on the time axis (x-axis) give the retention time and can be used to identify the components of the sample. The peak areas (on the y-axis) provide a quantitative measure of the amount of each analyte [100]. The retention time is directly related to volatility and polarity and thus to the interaction of the compound with the stationary phase [39]. For example, a lower retention time means a more volatile compound.

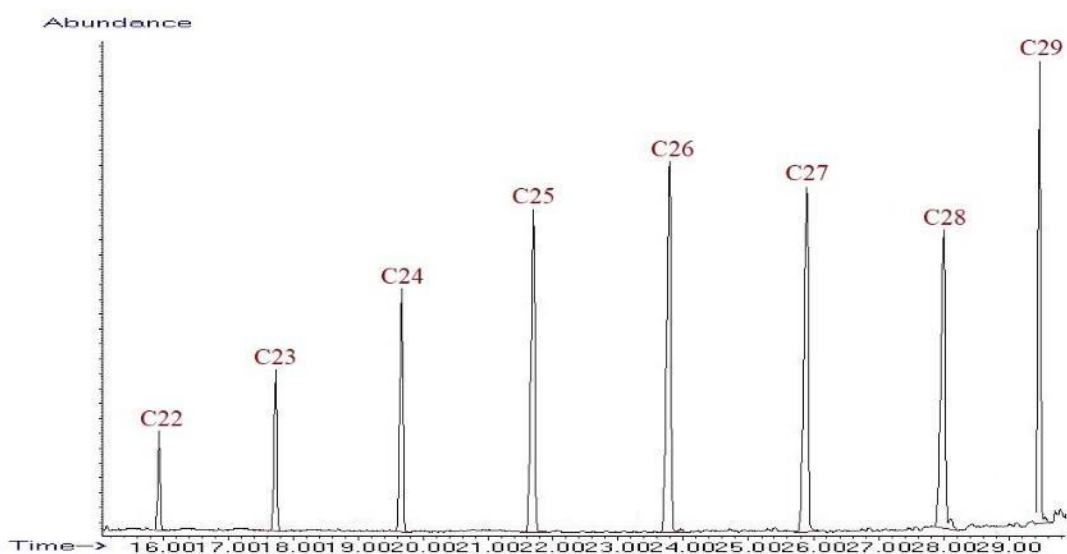


Figure 2.2: Chromatogram of cuticular hydrocarbons showing relative peak areas on the y-axis versus retention time (minutes) on the x-axis

2.3.2 Instrumentation

2.3.2.1 Carrier Gas

The standard primary instrumentation of a GC system is the injector port, column, oven and detector. The schematic diagram of a gas chromatography system is given in Figure 2.3. The carrier gas selection for GC is one of the most critical aspects that determine the performance of the system. There are two basic principles when selecting the correct carrier gas for analysis: it should be available at an affordable cost for analysis, and an optimum gas should be chosen to provide improved performance for a specific task [102]. Although helium is widely used as a carrier gas, argon, nitrogen and hydrogen gases can also be used for this. Generally, nitrogen is used as the carrier gas when a flame ionisation detector is used, and helium is used when a mass spectrometer is coupled to the GC [100].

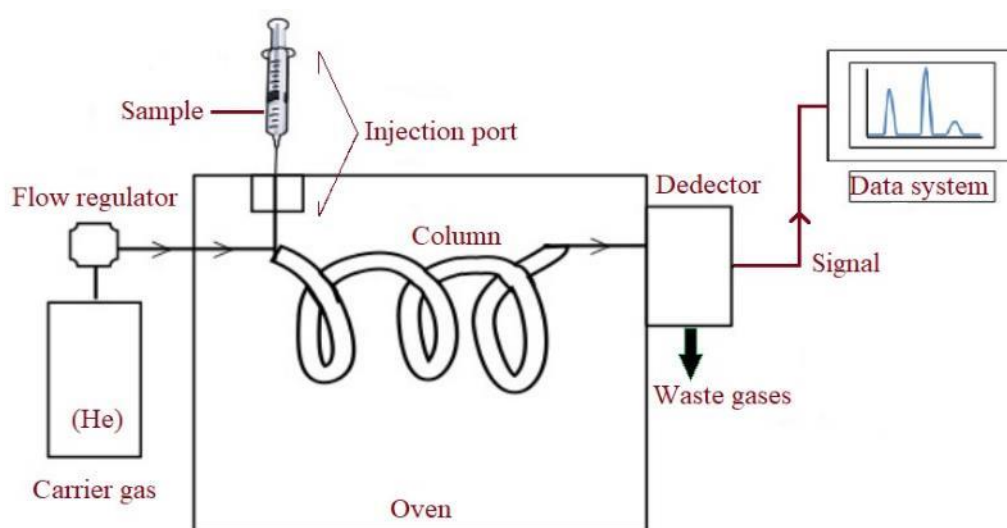


Figure 2.3: Schematic diagram of a gas chromatography system (GC) [101]

2.3.2.2 Sample Injection System

A syringe is needed to introduce the sample into the GC. This syringe can be operated manually or with an autosampler. While manual injection is rarely preferred in forensic laboratories, automatic injectors and autosamplers are widely used for repeatable sample injections [102]. The automatic injector syringe takes the sample from the vial and injects it through a rubber or silicone septum and into a heated sample port located at the head of the column. Generally, the initial temperature is kept at least 10°C below the lowest

boiling point in the analyte. Injection volumes can range from 0.1 μL to 200 μL , depending on the syringes. For higher column efficiency, it is necessary to use a suitable volume of samples. Slow injection or overloading the column with the sample mixture may cause poor resolution and broad peaks [100].

2.3.2.2.1 Split/Splitless Injector

Sample amounts for regular packed analytical columns range from a few tenths of a microliter to 20 μL . In capillary columns, smaller samples are used (by a factor of 100 or more). A sample splitter is usually needed to disperse a known small fraction (1:100 to 1:500) of the injected sample; the rest is not used and is discarded. A split/splitless injector scheme is shown in Figure 2.4. The split flow ratio to the column flow rate is known as the split ratio; the most common ratios are 50:1 to 100:1. For example, at a partition ratio of 50:1, one part of the injected sample enters the column, while the other 50 parts are discharged into waste through a trap. Almost all commercial GCs contain splitters and are also suitable for splitless injection use with packed columns. If too much sample matter is loaded onto the instrument, this will cause a poor peak shape and overload the detector; conversely, insufficient sample quantity causes poor method sensitivity, and the peak cannot be obtained as it cannot be detected at the detector [102]. If the analyte concentration is too low, as in trace analysis, the injection is performed in splitless mode [103].

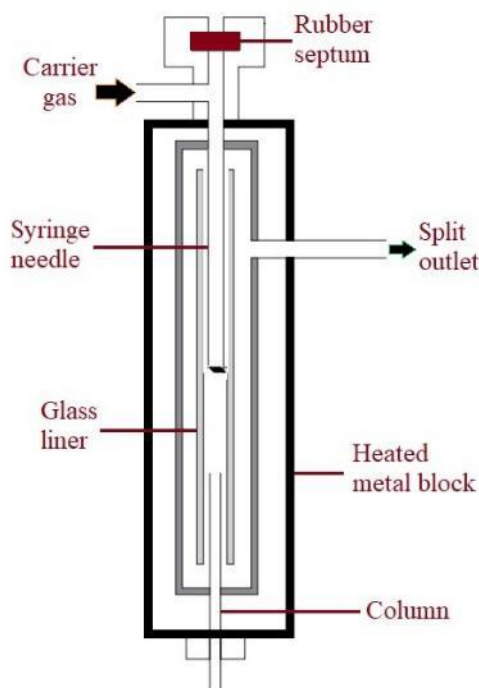


Figure 2.4: A split/splitless injector scheme [102]

2.3.2.3 Column

The main part where separation occurs in a GC analysis is the column. Chromatography column lengths generally range from 2 to 60 m, although longer columns are also available. It is possible to improve separation efficiency by increasing the column length. Columns are made of stainless steel, glass, fused silica or Teflon. Since they will be placed in an oven and be exposed to higher temperatures, columns are formed in coils with 10-30 cm diameter. There are two types of columns that can be used in GC: packed and capillary columns. Packed columns are produced by adsorption of the stationary phase as a thin liquid film to the surface of a finely divided, inert solid support, while capillary columns are produced by coating the inside of a capillary tube as the stationary phase in the form of a liquid film with one-tenth of a micrometre thickness.

Previously, packed columns were used in almost all GC analyses. However, over the years, capillary columns, which are much thinner than packed columns, have demonstrated their advantages over packed columns in terms of both speeds of analysis and column efficiency [104]. Before introducing capillary columns, using packed columns entailed reducing the large volume of carrier gas eluting from the GC, using various jet, membrane and effusion separators. However, since capillary columns are used

in GC devices, there is no need to use such separators [100]. Although capillary columns offer much higher separation power of volatile substances and many drugs in forensic laboratories, packed columns are used for ethanol analysis in alcoholic beverages [105]. There are four essential criteria to consider when choosing a column for separation in GC: the stationary phase, the inner diameter of the column, column length and the film thickness of the stationary phase. Each of these criteria significantly affects separation ability [102].

Capillary columns are also called open tubular columns because of the open flow path in them. There are two types of open tubular columns: wall-coated open tubular (WCOT) and support-coated open tubular (SCOT). WCOT is created by coating capillary tubes with a thin layer of liquid stationary phase. The SCOT column is obtained similar to a WCOT column, but it is coated with several times more stationary phases and has a larger sample capacity [106]. On the other hand, a WCOT column is more advantageous than a SCOT column in terms of column efficiency. However, if a SCOT column is compared to a packed column, the SCOT column will be more efficient [100].

In order to have a reasonable retention time in the column, an analyte must have compatibility with the stationary phase; thus, the principle “like dissolves like” is applied to express the polarities of the analyte and stationary phase [106]. For an effective separation, the stationary phase polarity must be equal to the polarities of the compounds to be separated. It is necessary to choose a polar stationary phase for polar compounds or a non-polar stationary phase for non-polar compounds. The interactions between a non-polar compound and a non-polar stationary phase are controlled by Van der Waals forces. In contrast, the interactions between a polar compound and a polar stationary phase are controlled by a dipole, π - π and/or acid-base interactions [102]. Polar stationary phases contain functional groups such as -CN, -CO and -OH. Saturated hydrocarbons, hydrocarbon-type stationary phases and dialkyl siloxanes are non-polar, while polyester stationary phases are very polar. Alcohols, acids and amines are included in polar analytes, while ethers, ketones and aldehydes are among the medium polarity solvents [100]. If the interaction of the organic solvent with the stationary phase is minimal, it is seen that it moves much faster through the column [102].

The separation order is determined by the boiling point of the compounds in the analyte. There is a linear relationship between molecular weight and boiling point [39]. Based on

this information, a hydrocarbon with a lower molecular weight will have a lower boiling point. Consequently, it will leave the column earlier than other compounds with higher boiling points [41]. In commercial columns, bound or cross-linked stationary phases are used. The purpose of this is to be able to rinse with a solvent when the stationary phase film surface becomes contaminated; thus, obtaining a longer-lasting column. When untreated columns are used, a small amount of the stationary phase leaves with the mobile phase during the separation process; therefore, its structure gradually deteriorates, which is called bleeding [107]. If a column is rinsed with a solvent to remove contaminants within the column, bleeding is further increased. This bonding and cross-linking structure prevents bleeding and extends the lifespan of the column [100].

2.3.2.3.1 Column temperature

The columns are formed in coils and are placed in an oven where they are exposed to a thermal programme. The column temperature is an important variable that must be controlled very comprehensively for precise and reproducible elution. Therefore, the column is modified with a thermostat oven, which must be able to provide the desired temperature range, from ambient temperature to 400°C or more. The temperature of the oven must be precisely and specifically adjustable [100]. The column oven has two temperature setting modes, isothermal GC and temperature-programmed GC, both of which directly affect separation ability. The column oven remains at a predetermined constant temperature throughout the chromatography analysis in the isothermal operating mode. In temperature-programmed GC, the column oven temperature increases or remains at certain temperatures at certain time intervals during the analysis. The isothermal method is preferred if a sample containing a relatively simple compound mixture with similar retention times is being analysed. For more complex analyte mixtures in the sample, using an isothermal mode may not provide effective separation for all components [107]. In such cases, using temperature-programmed GC provides better resolution and peak shape, as different temperatures will be applied for each fragment. At higher temperatures, sample compounds spend less time on the column, which lowers retention times, affecting the resolution. Generally, the optimum resolution is achieved at minimum temperatures [108]. However, reducing the temperature raises the elution time, so more time is required to complete the analysis.

2.3.2.4 Chromatography Detectors

The detector responds quickly to gas-phase compounds passing through the column and converts and records the incoming data into a measurable form. The combination of GC with a powerful detector makes it an indispensable device among analytical techniques in forensic sciences as well as in all disciplines. The ideal detector to be combined with GC is expected to have the following features [100,109];

1. Wide sensitivity range (10^{-8} to 10^{-15} g solute/s)
2. Stable and repeatable results
3. Linear response to solutes that exceed the order of magnitude
4. Wide temperature range (25°C-400°C, more)
5. Short response time independent of flow rate
6. Higher reliability and ease of use
7. Selective and sensitive response
8. Non-destructive to sample

Unfortunately, no commercially available detectors have all of these features in one package, and the most suitable detector for particular applications is selected based on the analytes of interest. The most widely used detectors are flame ionisation detectors (FID), thermal conductivity detectors (TCD), electron capture detectors (ECD) and mass spectrometer (MS). In addition to these, thermionic detectors, electrolytic conductivity detectors, photoionization and Fourier-transform infrared spectroscopy (FTIR) detectors can also be used [101]. For example, if the purpose of the analysis is to determine the alcohol (ethanol) in alcoholic beverages, MS is unnecessary because the analyte is well defined, the retention time is well known, and there will be very few interfering peaks in the chromatogram; therefore, FID can be used. If drugs are analysed, it is necessary to use mass spectrometry, as there may be other chemically similar compounds in the sample [102].

2.3.2.4.1 Flame Ionization Detector (FID)

FID is one of the most common detectors with GC, whereby the eluent leaving the column is burned in a small air-hydrogen flame. Most organic compounds generate ions and electrons when pyrolyzed at the air-hydrogen flame temperature, which is detected by monitoring the generated current by collecting the ions and electrons emerging in the flame. The resulting current ($\sim 10^{-12}$ A) is measured with a precision picoammeter [101]. FID is an accurate and easy-to-use detector with the advantages of higher sensitivity ($\sim 10^{-13}$ g/s), wide linear response range ($\sim 10^7$) and low noise [100]. It is the most useful detector for analysing most organic samples, even if they are contaminated with water and the oxides of nitrogen and sulphur.

However, functional groups such as carbonyl, alcohol, halogen and amine give less or no ions in the detector flame. Furthermore, it is entirely insensitive to non-flammable gases such as H₂O, CO₂, SO₂ and NO_x. The main disadvantages are that the FID destroys the sample during the combustion phase, requires additional gases and controllers and is a non-selective detector [101]. Although the two compounds, particularly the two isomeric hydrocarbons, have very similar retention times, they are different and can lead to false results in analysing hydrocarbons with GC-FID [39]. FID is widely used in forensic science to examine debris, food and fragrance [102].

2.4 Mass Spectrometry (MS)

MS is the most widely used detector for GC; Figure 2.5 shows the basic components of a mass spectrometer. MS gives quantitative data on the quantity of compounds present in the sample like other detectors. In addition, it can determine the chemical composition of an unknown compound in natural and biological systems. For this, the mass spectrum of the unknown compound must be compared with the database or the mass spectrum of a known compound standard [102]. The compound identification and structure determination feature of GC-MS makes it an invaluable combined tool for forensic analysis and many other analytical applications.

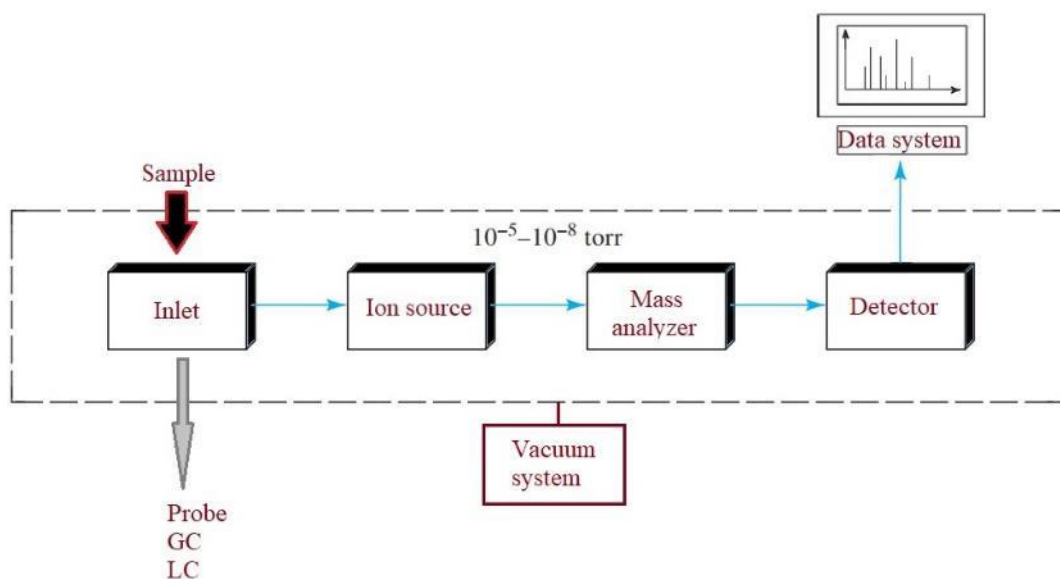


Figure 2.5: Schematic diagram of the essential components of mass spectrometry (MS) [100]

MS repeatedly scans masses during an analysis. For example, if a chromatography analysis runs for 10 minutes and a scan is performed every second, 600 (10 minutes x 60 seconds) mass spectra will be recorded [100]. Since the amount of data increases as the analysis time increases, a computer data system is needed to process this amount of data, which can be analysed in several ways. First, the ion abundances in each spectrum are summed and plotted as a function of time to give a total ion chromatogram. Secondly, in the chromatogram, the mass spectrum at a particular time can be displayed to identify the compound emerging at that time. Finally, a single mass-charge (m/z) value is selected and monitored by chromatography analysis. This technique is known as selected-ion monitoring. The mass spectrum of ions selected during a chromatography analysis is called a mass chromatogram [100].

GC and MS are highly compatible systems, both requiring heat in the range of 200-300°C, working with gas-phase sample elutes and being able to run with small quantities of samples (in microgram or nanogram quantities). The only difference between the two systems is in pressure; the atmospheric pressure output of the GC must be reduced to a vacuum of 10^{-5} to 10^{-8} Torr for the MS inlet [101]. Pressure regulation is needed for the combination of the two systems; therefore, an interface is used.

There are two types of MS, molecular and atomic [110]. In molecular MS, samples enter from the inlet system into the evacuated area of the MS. The inlet system of molecular MS aims to inject a micro-sample amount into the ion source, where the components of the sample are converted into gaseous ions by electron, photon, ion, or molecule bombardment. In atomic MS, the ionisation source is outside the evacuated area and also acts as the sample inlet system. Sample ionisation requires thermal or electrical energy. For atomic MS, the ion source must be very energetic to convert the sample into simple gas-phase ions and atoms. In contrast, in molecular MS, the ion source is much less energetic; therefore, the sample is converted into molecular ions and fragment ions [100]. Another difference between atomic and molecular MSs is the systems in which they are combined. While atomic MS is coupled with inductively coupled plasma (ICP), molecular MS is the most used analytical device with the GC system [111]. For more information on atomic MS, see Hoffman and Stroobant [110].

2.4.1 Instrumentation

2.4.1.1 Ion source

An MS system has three main parts: an ion source, mass analyser and detector. As low pressure is required, a pumping system is also included. The task of an ion source in an MS is to create gas-phase ions. Analyte atoms, molecules, or clusters are converted into the gas phase and ionised. There are two methods of ionisation: electron impact (EI) and chemical ionisation (CI) [100]. In EI ionisation, as electrons produced from a heated cathode accelerate towards the anode, they collide with the eluent from the GC column and provide ionisation. In contrast, in CI, a reactive gas (such as methane, isobutene, or ammonia) is ionised by electron bombardment, and the resulting reactive gas molecular ion then reacts with a neutral molecule to produce a reactant ion. The reacted ion then interacts with the eluent from the GC column to provide ionisation [102]. CI generally provides better sensitivity depending on the chemical properties of the analyte.

Most drugs contain essential functional groups in a CI source that are easily protonated in the gas phase; thus, a simple mass spectrum dominated by a large-protonated molecule ion peak can be produced. For example, drugs containing higher electron affinity functional groups such as halogens can be selectively ionised by CI with very higher efficiency [112]. On the other hand, EI is the most used ionisation source for all GC-MS

due to the simple interpretation of mass spectra and no need for additional gas to be introduced [110]. EI is discussed here in more detail since it is the ionisation method utilised in this study.

EI creates detailed and compound-specific mass spectra, and there are more than 100,000 EI reference mass spectra in the literature. EI leads to extensive fragmentation; thus, it is considered a “hard” ionization technique, and the molecular ion will likely be fragmented and not always observed [110]. Electrons are propelled forward by an electrically heated metal coil (filament), and they “collide” with analyte molecules, causing an electron to be lost and forming a charged molecular ion $[M^+]$, which then fragments into a combination of cations and neutral fragments. After fragmentation, most ions have a charge corresponding to the loss of one electron during the process, and these ions provide structural data. The molecular ion (if present, although it is frequently fragmented) with the greatest m/z ratio represents the molecular weight of the analyte. Once the ions have gone through the ionisation source, they are propelled further into the instrument by the driving force of an ion repeller, a metal plate with a slight positive charge and into the mass analyser where they are analysed [40].

2.4.1.2 Mass analyser

After the gas phase ions are produced, they are separated according to their mass. The physical property of ions measured by a mass analyser is their mass-to-charge ratio (m/z) rather than their mass alone. Therefore, it should be noted that the apparent m/z values for multiply charged ions are fractional parts of their actual masses [110]. There are several different mass analysers available for GC-MS, the most common of which are quadrupole, ion trap and time-of-flight [102].

2.4.1.2.1 Quadrupole MS

Four stainless steel rods are positioned horizontally in quadrupole MS. Direct current (DC) and radio frequency (RF) voltages can be applied to the opposite rods simultaneously in the same combination. When the appropriate combination of DC/RF voltages is applied to the detector, an ion with a certain m/z ratio will pass through the quadrupole MS and be detected; at the same time, all other non-target ions with various m/z ratios are lost. When combined DC/RF voltages are rapidly changed, ions with

different m/z ratios pass through the mass analyser and are detected. Generally, the required mass range for GC-MS is between 0 and 400 amu (atomic mass unit). Quadrupole MS is the most widely used mass analyser in forensic science [102].

2.4.1.3 Detection

The ions are detected after passing through the mass analyser, and the detector converts this information into a usable signal, producing an electric current proportional to the abundance of these ions [110]. The electric current is converted into a signal and is visualised as a mass spectrum using appropriate software [102]. When an in-coming ion hits the detector, the energy from the impact causes the emission of secondary particles, such as electrons or photons, whose number depends on the energy or speed of the in-coming ion. If all ions are accelerated to the same kinetic energy, the detection sensitivity of ions with higher mass and lower velocity is lower than that of low mass and fast ions. In order to increase the detection sensitivity, the ions are subsequently post-accelerated before striking the detector. It is preferred that a detector has a linear response, low noise, short recovery time and minimal variations in transit time (narrow peak width) and higher efficiency for converting the energy of the incoming ion to electrons or photons [111].

MS has two operation modes: total ion current (TIC) and sequential ion mode (SIM). All total ion currents between 0 and 400 amu are monitored in the fast scan mode in TIC mode. To obtain a TIC chromatogram, the ion abundances in each spectrum can be summed and plotted as a function of time, similar to a traditional GC chromatogram [100]. In order to determine the unknown compound content, the mass spectrum produced can be compared to the mass spectrum of a known standard compound or a mass spectrum database. Once a set of compounds has been defined, it is possible to run the MS in SIM mode, whereby key ions for the separated compounds must first be selected in the TIC mode [102]. In SIM mode, all information about other components in the sample is lost, but the detector operating in this mode only measures the current of selected ions; thus, the integral sensitivity can be increased by approximately two orders of magnitude. SIM is often used in ecological and forensic studies that work with trace amounts of organic compounds [111]. The appearance of a peak of the characteristic ion selected at known retention time is used to confirm the presence of the compound in the sample. In contrast, the peak area is used for quantification.

Both TIC and SIM modes are widely used in forensic analysis. TIC mode spectrum matches the spectrum of a standard compound to achieve the most reliable identification of a compound. However, monitoring an analyte in SIM mode is more sensitive and can provide reliable identification if combined with a selective chromatography separation [112].

2.4.1.4 Data Systems

Today, almost all GC systems use personal computers or existing data systems to collect, store and analyse data and create a database. These provide ease of use in device control, viewing, interpreting and sharing data [110]. Using computers and data systems, basic chromatography calculations such as peak area, area percentage, quantity analysis, internal standard, external standard, and normalisation calculation can be achieved easily.

2.5 Gas Chromatography-Mass Spectrometry (GC-MS)

GC-MS is a very powerful and user-friendly analytical instrument [41]. GC-MS is widely preferred because of its short analysis time, efficiency, higher resolution, sensitivity, non-destructive samples, cheapness, reliability and relative simplicity [101]. GC-MS is an integral part of all environmental, food and flavours, aromas, petroleum, petrochemical, forensics, fuel chemistry and pharmaceutical laboratory analyses [102].

2.5.1 GC-MS in Forensic Science

The general purpose of forensic laboratories is to analyse physical and biological evidence, determine the content of unknown samples, qualify or estimate the purity of detected substances and determine similarities or differences between multiple samples. In addition to the advanced and highly sensitive instrumentation widely available today, the GC-MS device stands out as an indispensable part of forensic laboratories [105]. Forensic science is a broad-ranging and versatile field that encompasses various forensic concerns, such as drug detection, toxicology and crime scene investigations, such as identifying the chemicals utilised in fire scenes or determining the presence or quantity of diacetylmorphine in suspicious dust samples [102]. The GC-MS technique is frequently used as the primary analytical tool for the determination of drugs in biological samples, such as blood and urine, saliva, hair, fingernails and toenails, as well as the

identification of solid samples, such as the contents of the liver, part of the brain and stomach in the post-mortem setting by pathologists [105]. In addition to forensic laboratories, other laboratories where combined MS systems are widely used include environmental protection agencies that analyse contaminants in air and water, anti-doping laboratories that detect all types of banned substances and drug testing laboratories to detect the presence of illegal substances in various biological fluids [105].

2.5.2 GC-MS Analysis of Cuticular Hydrocarbons

Choosing an accurate method is a significant step for determining the composition of CHC profiles, which are inherent in insect cuticles. Chromatography and spectroscopic methods are widely used due to the non-polar structures of CHCs and their volatility [113]. Different CHC groups have different mass spectrums, which show the mass of the molecule as well as the mass of the fragmented ions in the molecule. Identification of molecules is greatly aided by the presence or absence of $[M^+]$. Due to the fact that it is unstable when it has undergone EI, it is not present in the spectrum for the majority of the time. The majority of the n-alkanes include M^+ , which is critical in defining the length of the chain. Branched methyl alkanes need fragmentation mechanisms to correctly identify the location and length of the methyl group on the chain. In addition, the mass spectrum of n-alkanes has a characteristic profile. Figure 2.6 displays part of the GC chromatogram of two alkanes, tetracosane (C₂₄H) and pentacosane (C₂₅H).

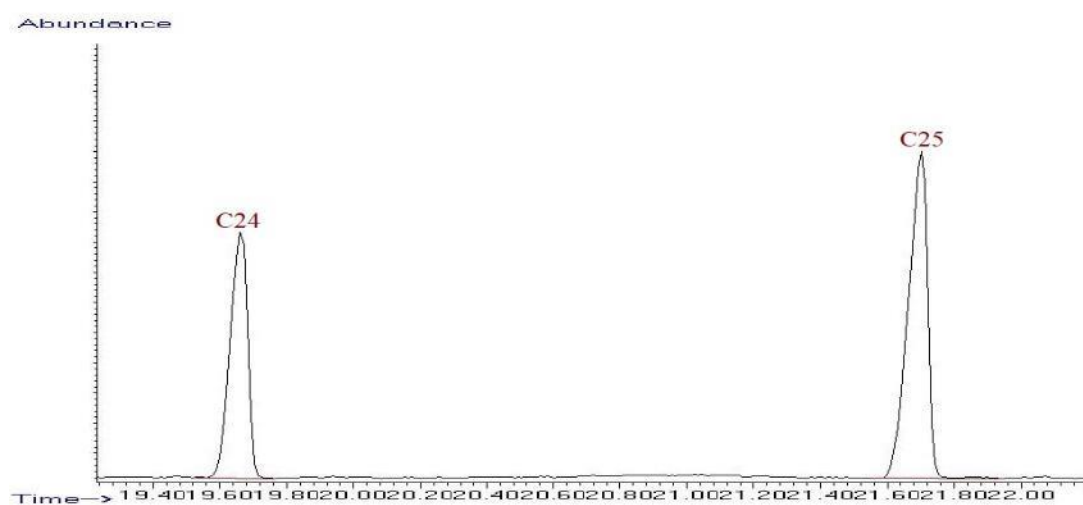


Figure 2.6: Part of GC chromatogram for tetracosane (C₂₄H) and pentacosane (C₂₅H) n-alkanes

The mass spectra of pentacosane ($M^+=352$) are shown in Figure 2.7. Alkanes are described by high-density m/z ratios at 43, 57 and 71 and decreasing ion densities of series, such as 85, 99, 113. These key ions are likewise found in the chromatograms of all n-alkanes, so the m/z ratio corresponding can only characterise n-alkanes with different chain lengths to the molecular weight of the n-alkane. Usually, these ions are more abundant than the fragment ions in the higher mass region [39].

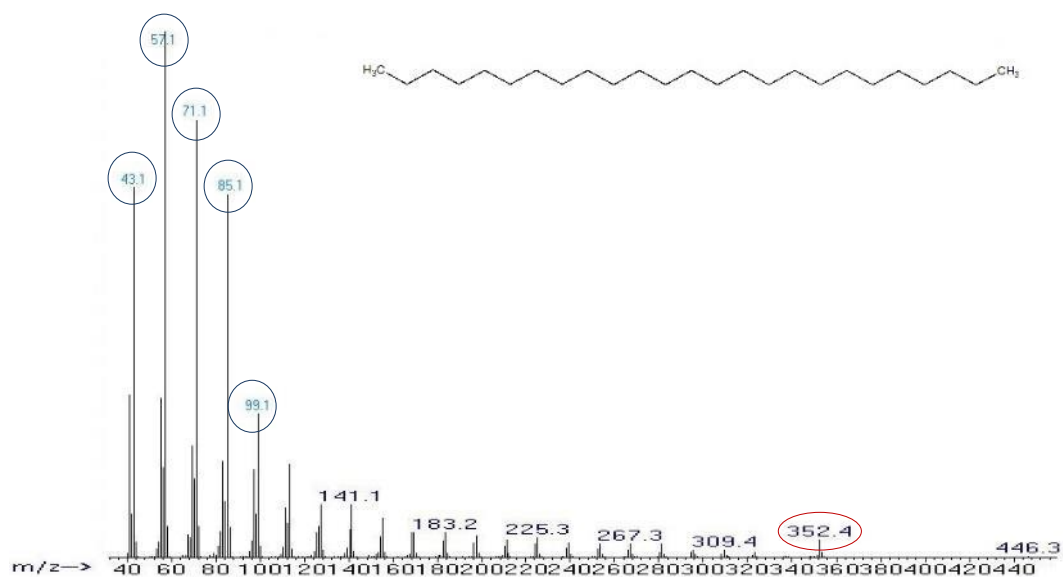


Figure 2.7: Example mass spectrum of pentacosane ($C_{25}H_{52}$) ($M^+=352$)

Alkenes also have a similar fragmentation pattern that allows them to be distinguished from other structures. There are two mass units difference between the molecular mass m/z ion values of alkenes and the corresponding alkane [39]. A mass spectrum of pentacosene alkene is given in Figure 2.8. Alkenes ion values of m/z at 41, 55, 69, 83 and 97 are present in higher abundance, followed by a decrease of m/z after the series 111, 125, 139.

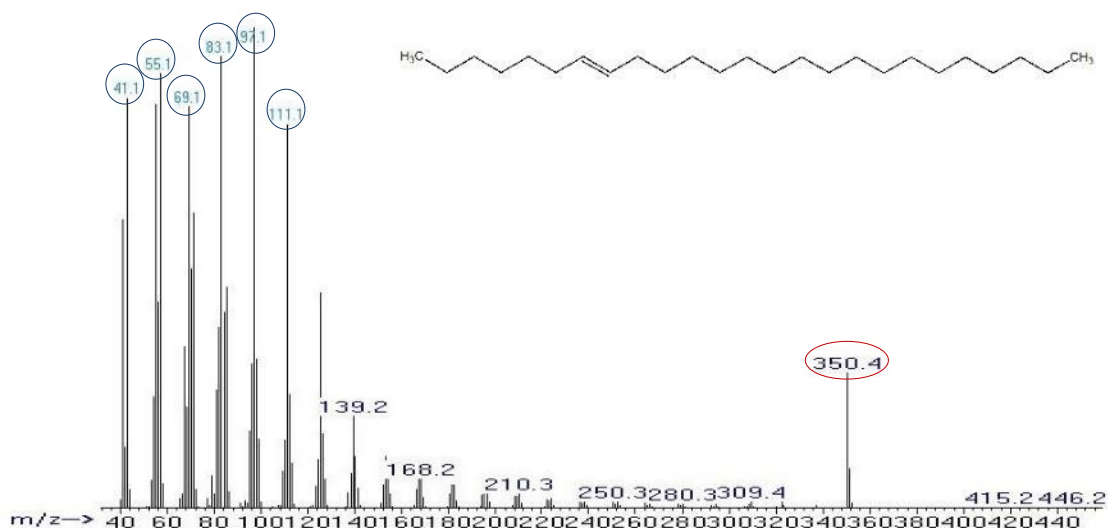


Figure 2.8: Example mass spectrum of pentacosene (C₂₅H) ($M^+ = 350$)

The location of the methyl alkane group can be determined using diagnostic ions in the mass spectrum. The diagnostic ions are positioned in branched methyl alkanes as a result of a fragmentation process termed Sigma-bond dissociation (σ). When n-alkanes are ionised, the length of the C-C bond is extended, which results in a reduction in the bond dissociation energy. This allows fragmentation to occur preferentially on more heavily substituted carbon atoms; the more abundant ion will be more stable in holding the charge, while the other fragment will become a radical. While the molecular ion is often absent in most branched methyl alkanes, an $[M-15]^+$ ion is almost always present, corresponding to the loss of the methyl group (M denotes the molecular ion, while 15 is the molecular weight of a cleaved methyl group $[CH_3]$). Branched methyl alkanes have a very similar spectrum to n-alkanes, but the characteristic mass spectra change with the position of the methyl group on the alkane structure. The higher abundance of an ion or pair of ions indicates the location of the methyl group on the linear chain.

The example in Figure 2.9 demonstrates how to identify branched methyl alkanes using the mass spectrum of 7-methylpentacosane (7-MeC₂₅) and shows two ion pairs present. Ion pairs are seen as a result of the loss of a hydrogen atom. The intensity of ions m/z 112:113 and m/z 280:281 exhibit greater abundance than the surrounding ions, and the fragmentation shows where the chain has cleaved, which is indicative of the position of the methyl group. Generally, the even mass ion is more abundant than the odd mass ion within these ion pairs. These stronger ions may be utilised to identify the location of the methyl group. An observed pattern is slightly altered when the methyl group is located at

the beginning of the carbon chain [39], in which case there will be no increased ions in the middle of the mass spectrum, but there will be two intense ions; one in the lower mass region and the other in the higher mass region, near the molecular ion. There is a similar pattern for dimethyl alkanes, except that each compound has four characteristic ion pairs.

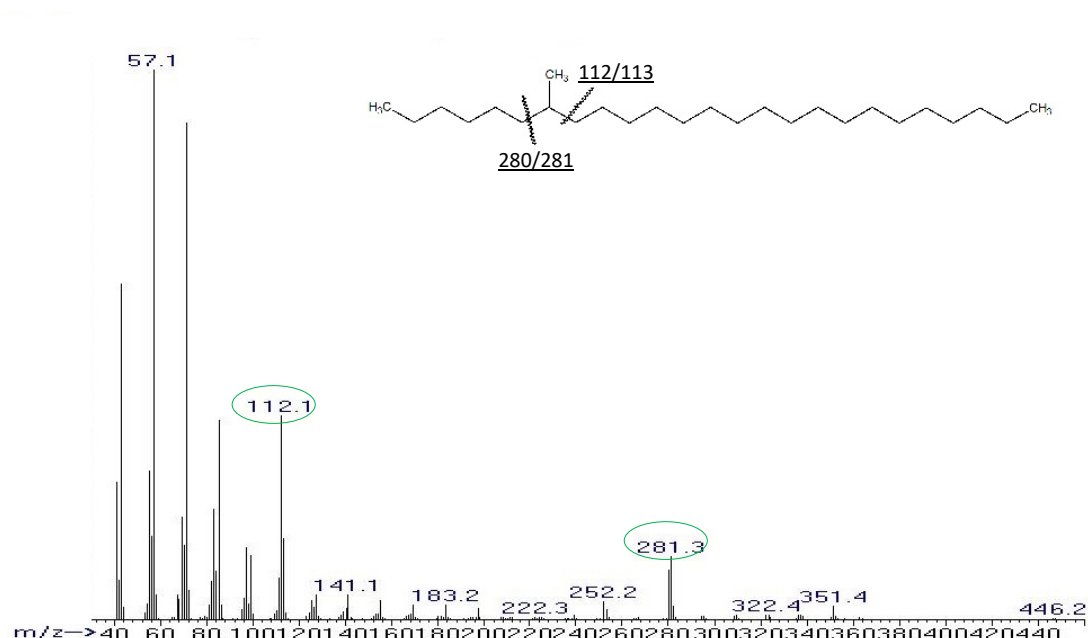


Figure 2.9: Example mass spectrum of 7-methylpentacosane (7-MeC₂₅)

2.6 Principal Component Analysis (PCA)

Clustering methods using PCA and multidimensional scaling (MDS) can be used with multivariate analysis techniques to reduce correlated datasets containing large samples, making them more manageable and revealing and classifying any trends or similarities in the data [41]. This is achieved by grouping the data obtained using the key components of the PCA or dimensions of the MDS according to statistically relevant correlations [40]. In the chromatogram of the CHC profile of insects, it is seen that retention times and peak areas give quantitative amounts of CHCs, and the relative peak areas in the chromatogram from each sample can be defined by a linear combination of principal components, each weighted by a specific set of numbers called loadings and scores [41]. The first principal component (PC1) represents the largest amount of variation in a data set, while the second and the other ordered PCs represent additional variations, each successively less significant.

Each PC has a corresponding value that represents the variance value as a percentage within the data set. The eigenvalue is used to define the number of key components to be used for the data set. It is optimal to define data with the least number of key components, as key variations become less important and lead to the exclusion of some data. Generally, four to six PCs are sufficient to describe the main variations. Thus, data sets are reduced from 100 data pairs to 4 or 6 predominant components. The output of a PCA calculation is presented as a scatter plot using the loadings of the two selected main components, generally PC2 and PC3 [40], whereby any clustering is visualised, grouped, and similarities or differences of samples can be easily described. Due to the complexity of the CHC profiles of insects and containing more than 100 compounds, it is not easy to visually observe the differences between insect samples of different species or ages. Moore and Drijhout [41] described some problems encountered in CHC analysis in this context:

1. Log-level variations in the percentage values of the individual compound.
2. The presence of a large number of zeroes.
3. Automatic data points correlation.
4. The “constant-sum constraint” property of composition data.

The total concentration of CHCs is distributed over a wide range of values for each individual, making it difficult to determine. In order to eliminate this problem, a normalisation process is applied to the data set. In certain studies, the absolute concentration of each compound (peak area) is divided by the cumulative concentration (total peak area) of all compounds, giving relative concentration by minimising the effects of higher variation within the process. For these compounds, the relative concentration is 0.5-40% of the overall profile. Compounds with very lower concentrations (<0.5%) are often excluded from the analysis, as they generally have a higher coefficient of variance [41]. PCA was chosen as the statistical analysis method for this thesis because of its capacity to classify clusters representing samples within the data and its user-friendly approach, including that it can be used with MS Excel.

Chapter 3

Materials and Method

3.1 Introduction

This chapter explains the concept of CHC analysis in detail, including the purpose of experiments to extract CHCs in the cuticle layer of insects, analyse them using GC-MS and statistically analyse and display the data generated. The procedure of experimental studies, collection and rearing of samples, materials used and GCMS analysis procedure is described.

3.2 Adult Fly Collecting

The colonies of *Lucilia sericata* and *Calliphora vicina* species were collected from the wild in Swindon (UK) (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). A redtop flycatcher trap was used (Figure 3.1), proposed during a verbal consultation with Dr Martin Hall, as redtop flycatcher traps are a very easy and effective method of collecting almost unharmed adult flies. To create the trap, the bottom of the plastic bag was cut off, and a wide-necked bottle was attached to the trap. The bag was then fixed between the lid and the bottle by cutting a hole in the lid. Fresh lamb liver weighing 200 g was placed in the bottle, then the trap was hung on a tree in a semi-shaded area and was left during the day. At the end of each day, trapped adult flies were identified and transferred to separate cages according to their species.



Figure 3.1: A redtop flycatcher trap

3.3 Adult Fly Rearing

L. sericata and *C. vicina* adult flies collected from the wild were used for oviposition. The adult flies were placed outdoors in rearing cages under ambient environmental conditions day and night (Figure 3.2). The adult flies were supplied with blood, sugar, water and dried milk powder. Fresh lamb liver was used as an oviposition medium placed on a petri dish in a box. The rearing method was similar to previous research [40], with the novelty of rearing the samples in fields under real environmental conditions.



Figure 3.2: Rearing cages in a) the UK and b) Germany, respectively

3.4 Larvae Rearing

Once blowfly eggs were laid on the oviposition medium, the petri dish containing the eggs and meat was transferred into small lunch boxes covered with muslin to circulate a good airflow. The boxes were placed in outdoor cages, and the weather temperatures were measured by an individual datalogger during all life stages. The floor of each cage had a small layer of sawdust and soil to allow for pupation. These samples reared in the fields were being referred to as outdoor samples. For empty puparia examinations, *C. vicina* experimental sets were reared outdoors in cages with adult flies without direct exposure to natural weather conditions. On the other hand, *L. sericata* empty puparia were buried in soil and exposed to environmental conditions such as wind, rain and sunlight. In addition, control groups were formed for both species. For the control groups, although all experimental stages were the same, they were reared in a $25\pm 1^{\circ}\text{C}$ incubator in the laboratory instead of outdoors. Control groups are referred to as “indoor samples”.

3.5 Sample Preparation

The liquid extraction method described by Moore [40] is preferred for CHC profile extraction because it is a simple and inexpensive method that demonstrably gives successful results. This sampling method was applied to the life stages and empty puparia of *L. sericata* and *C. vicina* blowfly species. Ten replicates were always taken for each sample set during the extraction process to compensate for any variation.

3.6 Extraction of Cuticular Hydrocarbon Profiles

CHC analysis begins by extracting CHC profiles from the insect cuticle by liquid extraction (dissolving CHCs in an organic solvent). As the hydrocarbons are non-polar, a non-polar solvent, such as hexane or dichloromethane, is preferred for extraction. Polar solvents, such as methanol or ethanol, can predispose insect samples kept in solvent for too long to the extraction of many internal (polar) molecules [41].

3.6.1 Blowfly eggs

After female adult blowflies lay eggs in the oviposition medium, the meat was removed from the rearing cage, and the eggs were carefully collected without damage and were placed in a clean 2 mL GC vial. Liquid extraction with hexane was used to remove CHCs from the cuticle of the blowfly eggs. Hexane (100 μ L) was added until the eggs were completely submerged and left for 10 to 15 minutes. The hexane extract containing the CHC profiles was then transferred to a clean vial directly. Finally, the hexane was left to evaporate and left to dry down completely. All samples were stored in the refrigerator at 4°C until they were required for GC-MS analysis. Samples were labelled Day 0 (E0).

3.6.2 Larvae

The larvae were pooled together to ensure that the percentage concentration was sufficient for GC-MS analysis. For the first instar stage, nearly 20 larvae were used. When the larvae age, they increase in size so that the amount required for extraction is reduced. The larvae were extracted daily upon emergence from the egg to pupation. For the extraction, larvae were placed into a 2 mL GC vial with hexane, ensuring that they were fully submerged and left for 10 to 15 minutes (Figure 3.3-a). If the sample is kept in the solvent for too long, the polar compounds are extracted along with the non-polar compounds [39]. Clean extracts can usually be obtained from larvae collected from plants or their nests. However, if the larvae are collected from the meat source, some tissues may accumulate on them and contaminate these extracts. A small column with silica gel or on a TLC plate can separate polar from non-polar compounds (Figure 3.3-b). Also, saturated and unsaturated hydrocarbons can be separated via a silica gel column impregnated with silver nitrate. The larvae were the only life stage requiring column chromatography [39].

The column was prepared using a Pasteur pipette plugged with glass wool and coated with about 1 cm of silica gel. The column was first wetted with 100 μ L of hexane, then the hexane extract containing the larval CHC profile was transferred onto column chromatography. Finally, an additional 500 μ L of hexane was added. The eluted hexane was collected into a clean vial. Then the hexane extract was left to evaporate entirely. All samples were stored in the refrigerator at 4°C. Extractions of first instar larvae hatching from eggs were labelled as Day 1 (D1).

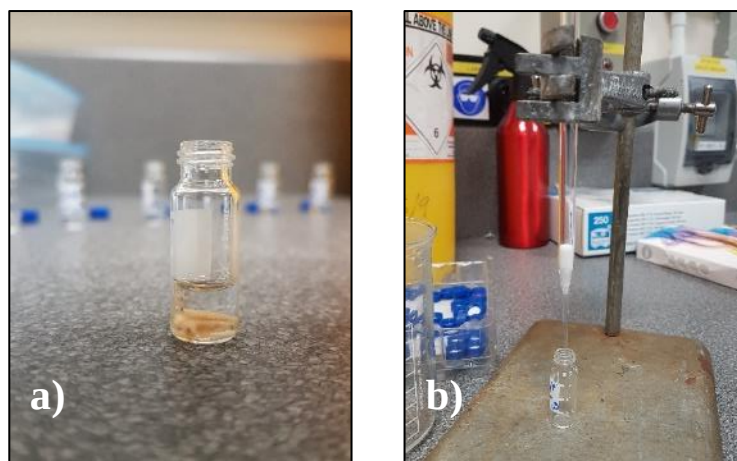


Figure 3.3: Extraction steps a) submerged larvae in hexane, b) column chromatography

3.6.3 Puparia

Puparial extractions for *L. sericata* and *C. vicina* were carried out similar to the larvae samples. The CHC profiles were extracted using two pupae per sample and were performed one day apart until eclosion. As pupae are closed structures, they were drilled with the help of a needle before being placed into the hexane. For the extraction, pupae were placed into a 2 mL GC vial with hexane, ensuring that the pupae were fully submerged (300 μ L) and left for 10 to 15 minutes. The hexane extract containing the puparial CHC profile was transferred to a clean vial directly, then the hexane was left to evaporate. All samples were stored in the refrigerator at 4°C. Samples from the first day of the puparial stage were labelled Day 1 (P1).

3.6.4 Empty puparia

The CHC profile extractions of the empty puparia were carried out using the same methodology as the puparia extractions. The CHC profiles were extracted using two empty puparia per sample. Samples from the first day of the eclosion were labelled Day 1 (E1).

3.6.5 Adult flies

The CHC profiles of the adult flies were extracted on the first day after the puparial stage ended, and adult flies emerged. The adult flies for the experiment were maintained in rearing cages before extraction. The extractions were carried out with the same methodology as the puparia extractions, in that one adult fly per vial was placed into a 2 mL GC vial and submerged with hexane (500 μ L). The extractions were labelled as Day 1 (A1). The extractions were completed for Day 1, Day 2, Day 3, Day 4, Day 5 and Day 10.

3.7 Chemical Analysis: Gas Chromatography-Mass Spectrometry

The dried extract was reconstituted in 30 μ L hexane before GC-MS analysis. Chemical analysis of all extracts was carried out on an 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 μ m film thickness) coupled with an Agilent 5973 Network Mass Selective Detector. The GC was connected to a computer, and the data was processed with Agilent Chemstation software. Elution was carried out with helium at 1 mL/min. The oven temperature was programmed to be held at 50°C for 2 minutes; then it was 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 to 320°C at 20°C/min, where it was held for 2 minutes. The mass spectrometer was operated in Electron Ionisation at 70 eV, scanning from 40-500 amu at 1.5 scans/s. CHCs were identified using a library search (NIST08). The percentage concentration was calculated by computing each peak area, which produced a percentage total peak area of all compounds in the sample for each species. Unfortunately, no information on alkene double bonds could be gathered due to the small sample size and volume.

3.8 Chemicals

Hexane (HPLC grade) was purchased from VWR Chemicals and was used for extracting CHCs from the epicuticular layer. Silica gel was purchased from Acros Organics (60-200 micrometre, 60A) and was used in column chromatography to separate the non-polar compounds from the polar compounds present in the insect hexane extracts.

3.9 PCA

Statistical analysis was performed using multivariate add-ins a Microsoft Excel written by Tom Thurston, using an original development by Les Erskine [114], as previously used by Moore [40]. For PCA, peak areas on chromatograms were identified using Agilent Chemstation software, focusing mainly on CHCs. All data were normalised before the PCA calculation. The n-alkanes were generally studied from C18:H to C34:H. All CHCs with trace amounts and peak area less than 0.5% were excluded from the PCA data sets. All possible PC combinations were plotted until the best separation was obtained between sample extractions. NCSS 2020 software was used to create the 3D graphic plane with the dataset obtained by PCA. PC2 and PC3 scores were used when generating the graphs, as they gave the best separation and ensured consistency between the results. While creating 3D graphics, another score was chosen as the third axis, which provides the most apparent distinction among others. For age estimation, it is expected that a set of clusters will appear on the PCA chart for each day, and it is desirable if sample clusters are separated from each other as smoothly as possible. However, accumulation is a predictable problem as the number of samples in the graph increases. Therefore, it is essential to analyse repeatable samples to distinguish any variation due to chemical variation from the natural random variation in these loadings or scores.

Chapter 4

Species Identification Using Cuticular Hydrocarbons

4.1 Introduction

The first step in calculating the PMI_{min} in forensic entomology is determining the species of entomological evidence. Different insect species have different life cycle timings and respond differently to the environmental conditions to which they are exposed [39]. Consequently, species identification is important, and it enables a more accurate determination of entomological data regarding development time [115]. Generally, morphological methods are used for species identification, whereby the morphological characters of insects are compared with published standard keys to identify the species and estimate the possible age [116]. However, there are few identification keys in the literature that describe Calliphoridae [36–38,117]. Also, determining the species of the relevant insects at the immature larvae stages requires experience and special taxonomic knowledge.

In cases where identification cannot be accomplished using the morphological method, the first instar larvae must be reared until the adult fly stages because although identification can sometimes be established in third instar larvae, their identities are always confirmed in the adult stage. This is detrimental to time efficiency during forensic investigations and criminal proceedings. Furthermore, such methods require rearing equipment and some entomology knowledge; a failure of larvae rearing can cause serious problems [16]. On the other hand, CHC analysis provides a quick and accurate complementary alternative for species identification that is simple, feasible and cost-effective.

The qualitative and quantitative differences that arise between CHC profiles of various species make them species-specific in all insects [55,79]. Each insect species has a unique CHC profile created from different combinations of CHCs [32]. Forensic entomological studies have successfully demonstrated the use of CHC analysis for species identification for bees [118], ants [119], termites [120], blowflies [32,78], flesh flies [45], cockroaches [121,122], wasps [123], beetles [124], mosquitos [125] and grasshoppers [126]. These results were obtained mainly by GC-MS analysis with multivariate statistical methods, such as discrimination analysis (DA) or PCA.

CHC analysis has the potential for the identification of Calliphoridae species, especially for life stages such as eggs, first instar larvae, adult flies and their empty puparia, which are very difficult to characterize morphologically [32]. Previous studies describing CHC compounds in Calliphoridae have led to the discovery of specific CHC profiles originating from sex and developmental stage differences. Goodrich [127] was the first researcher to investigate the chemical composition of the cuticular layer of adult male and female *Lucilia cuprina* blowflies and the empty puparia using thin-layer chromatography (TLC).

CHCs are stable compounds and do not evaporate at ambient temperatures due to their long chains, so their degradation is slow. Therefore, the stability of CHC profiles provides the potential for species identification even years after museum samples were collected [45]. The chemical profiles obtained from broken empty puparia or insect parts can also be used for species identification. Moore et al. [45] performed species identification using the CHC analysis for eleven Sarcophagidae adult fly species using a customized extraction protocol adapted to fixed museum samples. They also distinguished males and females of the same species. Moreover, using museum samples, they demonstrated the stability of CHCs extracted from samples collected many years ago. This study again showed that CHC analysis is a non-destructive technique, and samples can be returned to collections undamaged after analysis.

This chapter focuses on species identification for blowfly eggs, first instar and post-feeding larvae, pupae and empty puparia of *Lucilia sericata* and *Calliphora vicina* species, collected and reared outdoors, using CHC analysis with GC-MS. The adult fly stage is not included in the species identification because adult flies of *C. vicina* are metallic blue, while *L. sericata* is bright green (seen in Figure 4.1). They can be easily

distinguished from each other by their colours. Multivariate statistical analysis will create PCA plots to interpret and visualise the results obtained. For the research, colonies were obtained from blowfly eggs collected from the wild and reared outdoors in the UK. They were exposed to environmental effects to test the stability of the CHCs, to ensure that identification could still be achieved.

4.2 Egg Stage

Identification of the blowfly egg stage is difficult because the morphological characters are very similar (Figure 4.1). Greenberg and Singh [70], Sukontason et al. [73], and Giffen et al. [68] performed species identification by examining the morphological characters of blowfly eggs using SEM, but this is an uncommon device and technique in forensic laboratories. Moreover, identification through the characteristics of blowfly eggs requires a complex morphological examination by an experienced forensic entomologist or taxonomist [49].

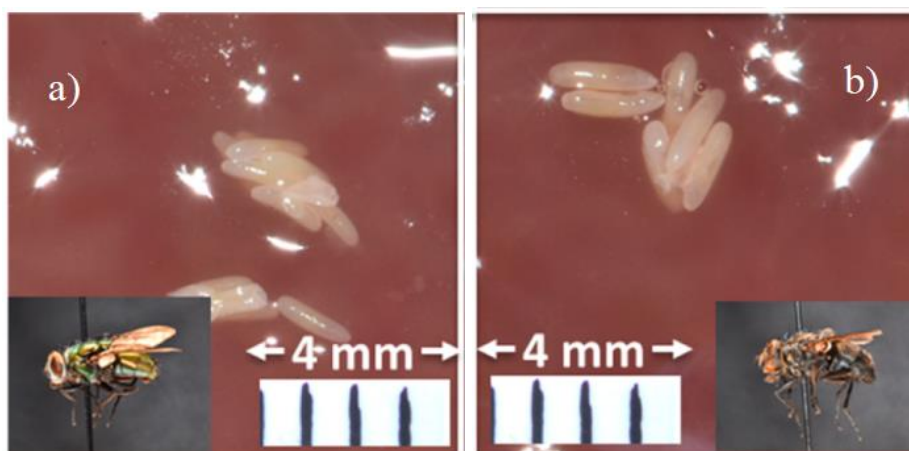


Figure 4.1: Images of blowfly eggs obtained from Calliphoridae family a) *L. sericata* adult fly and eggs b) *C. vicina* adult fly and eggs [68]

Moore [40] researched the species identification for the blowfly eggs and empty eggshells of Calliphoridae species. In addition, Lunas et al. [128] demonstrated the CHC profiles and fatty acid composition of eggs from blowfly species could be utilised as an additional taxonomic tool to expedite forensic knowledge. They used constant temperatures and controlled laboratory conditions for the rearing process. However, the stability of CHC on blowfly eggs collected from the wild is unknown.

This study aimed to identify the species using the CHC analysis on the chemical profiles of *L. sericata* and *C. vicina* blowfly eggs collected from the wild. Results were visualised by applying PCA.

4.2.1 Results for Egg Stage

The blowfly eggs of *C. vicina* and *L. sericata* adult flies collected from the wild were extracted, and their chemical profiles were analysed by GC-MS. The CHCs detected in the chromatograms are given in Table 4.1, with the total percentage peak area and percentage standard deviation of each compound. The chain lengths of the CHCs detected range from C23:H to C33:H. The chemical profile of *C. vicina* contained 23 CHC compounds, and *L. sericata* contained a chemical profile of 33 CHCs. The data was used for the subsequent PCA. The peak areas of CHCs with insufficient concentration (<0.5%) were not included in the statistical analysis.

Table 4.1: CHC compounds extracted from wild *C. vicina* and *L. sericata* blowfly eggs, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>C. vicina</i>	<i>L. sericata</i>
1	Tricosene*	ND	1.28±0.59
2	C23	1.06±0.86	2.32±0.98
3	11+13-MeC23	ND	1.04±0.51
4	C24	ND	0.50±0.12
5	3-MeC24	ND	0.51±0.08
6	Pentacosene*	ND	5.11±2.03
7	C25	5.61±3.86	3.65±1.25
8	11+13-MeC25	ND	2.78±1.22
9	C26	1.30±0.53	0.95±0.17
10	4-MeC26	3.83±2.64	ND
11	3-MeC26	ND	1.01±0.33
12	Heptacosene*	ND	4.14±1.95
13	C27	18.41±11.99	13.76±2.93
14	11+13-MeC27	1.42±1.06	1.00±0.34
15	3-MeC27	1.59±1.10	1.49±0.36
16	C28	3.11±1.99	2.50±0.49
17	4-MeC28	7.95±5.12	1.06±0.21
18	Nonacosene*	ND	1.12±0.41
19	C29	14.63±10.30	20.03±4.68
20	11+13-MeC29	11.27±7.89	5.92±2.01
21	9-MeC29	ND	2.36±0.75
22	7-MeC29	3.15±2.08	2.80±1.22
23	5-MeC29	0.73±0.51	ND

Peak No.	Peak ID	<i>C. vicina</i>	<i>L. sericata</i>
24	4-MeC29	1.51±0.83	0.98±0.37
25	3-MeC29	4.07±2.87	1.21±0.73
26	C30	0.87±0.64	0.75±0.28
27	8-MeC30	1.43±0.97	0.53±0.20
28	4-MeC30	3.07±2.24	ND
29	3-MeC30	ND	1.57±0.66
30	Hentriacontene*	ND	4.25±1.64
31	C31	0.83±0.67	1.93±0.58
32	13+15-MeC31	1.24±0.84	5.50±1.14
33	9+11-MeC31	5.19±3.50	5.38±0.79
34	3-MeC31	6.42±4.40	ND
35	7-MeC32	ND	0.77±0.13
36	5-MeC32	ND	0.63±0.25
37	C33	1.30±0.86	1.66±0.35

ND Not detected

* Double bond positions were not determined

Comparative GC-MS chromatograms are shown in Figure 4.2. In the chromatograms, peaks with retention times of 8 and 14 minutes were generally polar compounds. Since this study was mainly based on CHCs, these polar compounds were not included. The middle portion of the chromatogram was predominantly composed of CHCs with relatively lower boiling points ranging from C22:H to C25:H. As the retention time increased, longer carbon chains with higher boiling point CHCs were detected.

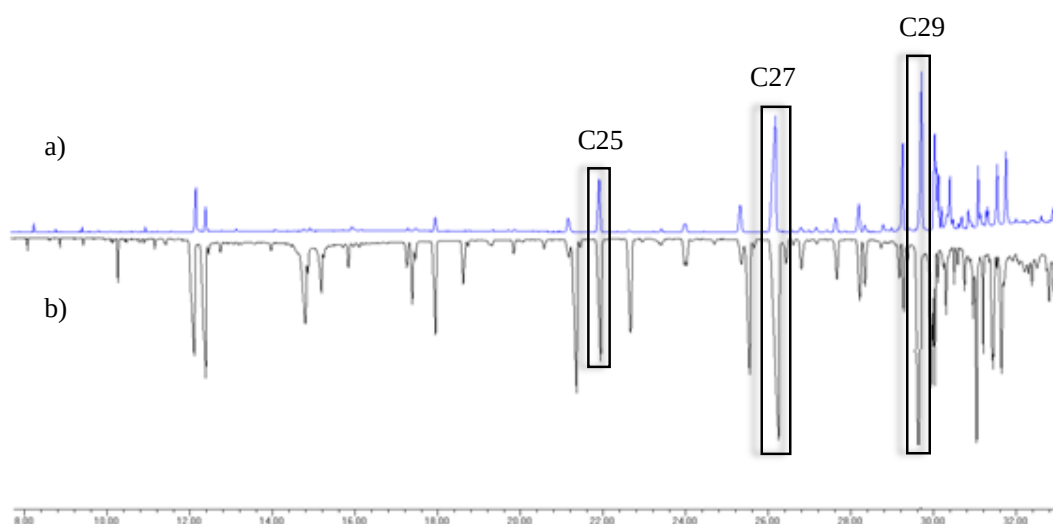


Figure 4.2: GC chromatograms showing CHC profiles of blowfly egg species, a) *C. vicina*, b) *L. sericata*. The marked areas show the species-specific key regions

The distribution of n-alkane, alkene and branched methyl alkane compounds by species are given in Table 4.2. Although the numbers and concentrations of n-alkanes detected in *C. vicina* and *L. sericata* eggs were similar, the numbers and concentrations of branched methyl alkanes and alkenes were significantly different for the two species. Despite the fact that *L. sericata* had a greater number of branched methyl alkanes and alkenes identified in its CHC profile, the percentage concentration of branched methyl alkanes in *C. vicina* was higher than in *L. sericata*. Furthermore, alkenes were not detected in *C. vicina* eggs. However, the alkene concentration in the chemical profile of *L. sericata* eggs was significantly higher. Five alkenes were detected in *L. sericata* eggs.

Table 4.2: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for blowfly eggs collected from the wild

	<i>C. vicina</i>		<i>L. sericata</i>	
	number	Total % peak area	number	Total % peak area
n-alkanes	9	47	10	48
Branched methyl HC	14	53	18	36
Alkenes	-	-	5	16
<i>Total</i>	23	100	33	100

The total number of branched methyl alkanes appeared to be higher than the n-alkane numbers in the chemical profiles; however, the total peak areas (concentrations) of the n-alkanes were greater than those of the branched methyl alkane. When the percentage concentrations of n-alkanes were examined, it was seen that C25:H, C27:H and C29:H (odd-chain length n-alkane, OLA) were predominant in the CHC profiles of both species. In *C. vicina* blowfly eggs, the predominant n-alkane was C27:H. On the other hand, C29:H was the predominant n-alkane in *L. sericata*.

It was observed that 11+13-MeC29 was the most abundant branched methyl alkane for both species, but its concentration was higher in *C. vicina* blowfly eggs than in *L. sericata*. Branched methyl alkanes 4-MeC26, 4-MeC28, 3-MeC29, 4-MeC30 and 3-MeC31 were specific for *C. vicina* eggs. In contrast, 11+13-MeC25 and 13+15-MeC31 were specific for *L. sericata*.

Species classification can be made according to the presence/absence or different ratios of percentage concentrations in CHC profiles; however, this process becomes more complex when the number of species and samples increases. Therefore, PCA was applied to the data presented in Table 4.1 to visualize any distinctions present within the chemical profiles of the two species.

All CHCs presented in Table 4.1 were applied for PCA using six principal components, describing 99.2% of the dataset variation within the first three components, comprising 89.7, 8.9 and 0.7% (PC1, PC2 and PC3), respectively. The number of repeats per species was 10 times ($n = 10$). In Figure 4.3, the plot was derived from the PC2 and PC3 scores, chosen among the six principal components because they clearly showed variation between species. Compounds that had significant scores played an essential role in the discrimination seen in the PCA plot. The main compounds with significantly higher scores were nonacosane (C29:H), 4-methyloctacosane and pentacosene. Thus, a clear distinction between the two species at the egg stage was obtained.

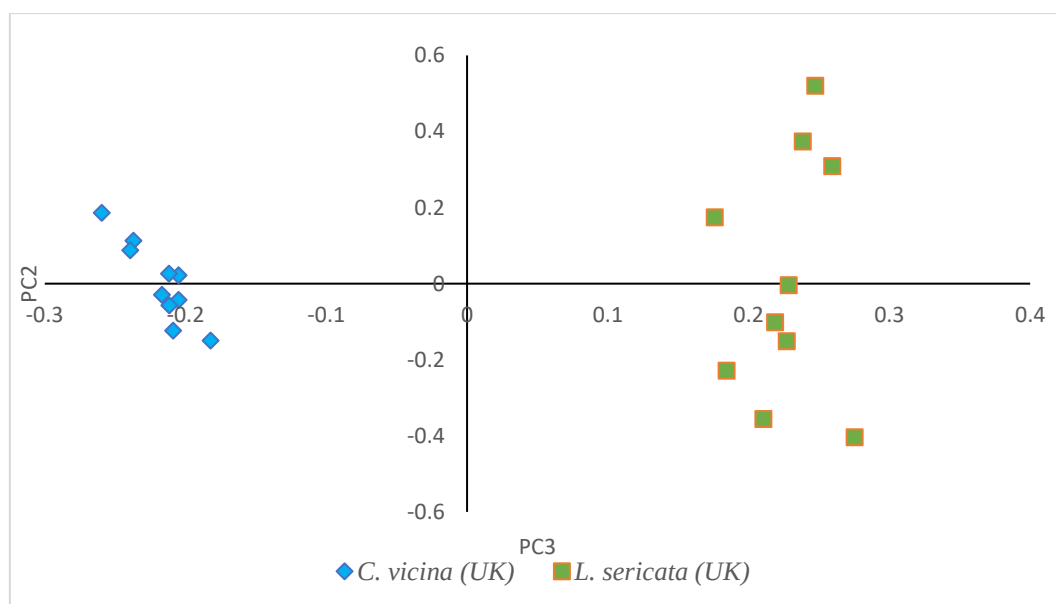


Figure 4.3: PCA plot showing PC3 against PC2 for *C. vicina* and *L. sericata* eggs collected from the wild, using n-alkanes, alkenes and branched methyl alkanes

4.2.2 Discussion of Egg Identification

The genetic coding of insects has a significant impact on the profile of CHCs, which also vary based on environmental variables [128]. The impact of species-specific, genetic and

environmental factors on the CHC profiles of blowfly eggs was evaluated in this field investigation. Species identification was performed between *C. vicina* and *L. sericata* blowfly eggs species collected from the wild using the CHC analysis and PCA. It is possible to distinguish between species by looking at these qualitative and quantitative differences in the CHC profiles of both species. However, this situation will get more complicated as the number of species and samples increases. PCA plots show slight variation and signify good experimental reliability.

CHC analysis combined with PCA is advantageous for PMI_{min} estimations as it facilitates quick species identification in a short time, including at early stages (such as the blowfly egg stage). Therefore, it has the potential to be a valuable tool for forensic investigations. In current identification techniques, identification is made by comparing the morphological characters of blowfly eggs with key characters in the literature via microscope or SEM imaging. However, this situation requires both morphological examination experience and time. Furthermore, the number of standard keys for species that have been published is very restricted in the literature.

These preliminary results include the CHC profile of only two species. Future studies involving more species are needed to expand the results and improve the method. The broad scatter seen in the PCA plots may be due to the small number of sample species chosen for this study.

4.3 Larval Stage

Larvae are the most common entomological evidence at a crime scene; therefore, they are the most used life stage in estimating PMI_{min} and provide the most helpful information. The mouthpiece (cephaloskeleton), spines, anterior and posterior spiracles are the morphological characters used for larval species identification [40]. SEM images, light microscopy photographs and line drawings are usually used to determine the morphological characters [38], but these techniques require both good imaging and expertise. Although previous studies can provide primers for species identification [34,37], the number of published morphological characteristic key lists for species is limited. Furthermore, in the early stages of the Calliphoridae developmental stages, the number of variable characters used for species identification is relatively lower, and early-stage immature larvae species identification is complicated due to their small size.

Few taxonomists can accurately identify first instar larvae. Szpila et al. [36] prepared a morphological characteristic key list for the first instar larvae of *C. vicina*, *Phormia regina* and *Lucilia illustris* species using the SEM imaging technique. In another research, Szpila et al. [38] determined the identification criteria according to the morphological characters of blowfly larvae belonging to the Luciliinae family. However, drawings or photographs showing morphological characters may be small and ambiguous, or taxonomically important characters may not be mentioned [117]. In some cases, DNA-based methods [129–133] can be applied successfully instead of morphological characters, but they are more expensive and complicated, requiring advanced experience, and a complete representation of all possible species is required for DNA sequencing [115].

CHC analysis shows the potential to be more advantageous than other identification techniques as it does not require larvae rearing until the third instar stage, and it is more cost-effective than DNA analysis. It can be applied to first and second instar larvae to obtain highly accurate results. Lockey [74,79,134] stated that closely related species have qualitatively similar and quantitatively different CHC profiles, while distantly related species tend to have different profiles, both qualitatively and quantitatively. It was also mentioned that CHC composition has proven to be particularly useful in separating insect species closely related to specific species that would otherwise be difficult or impossible to separate.

Roux et al. [135] analysed the CHC profiles of *Calliphora vomitoria*, *C. vicina*, and *Protophormia terraenovae* blowfly species by GC-MS and discriminant analysis during ontogeny. They found that CHC profiles can be used as phylogenetic markers and can identify species for all life stages, from the egg to the adult fly. Their results also showed that *C. vicina* and *C. vomitoria* species were closely related, while the *P. terraenovae* species were distantly related based on the CHC profiles. Moore [32] classified three blowfly species, *L. sericata*, *C. vicina* and *C. vomitoria*, for first instar larvae using GC-MS and PCA, finding that each species has a unique CHC profile and even 1-day old larvae, which are difficult to classify according to morphological criteria, could be identified by CHC analysis. However, these studies were conducted under laboratory conditions at constant temperatures; thus, the impacts of fluctuating temperatures on CHC profile stability in the larval stage are unknown.

This study aimed to identify species using CHC analysis on the chemical profiles of first instar larvae reared outdoors by exposure to fluctuating temperatures, visualising results by applying PCA.

4.3.1 Results for Larvae Stage

CHC profiles of *C. vicina* and *L. sericata* larvae reared outdoors were extracted on the first day of the larval stage (first instar larvae). Chromatograms of the first instar larvae are given in Figure 4.4. All CHCs detected are given in Table 4.3. The detected compounds were used for subsequent PCA analysis. The peak areas of CHC with insufficient concentrations (<0.5%) were not included.

Table 4.3: CHC compounds extracted from *C. vicina* and *L. sericata* first instar larvae reared outdoors, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>C. vicina</i> First larval day	<i>L. sericata</i> First larval day
1	C16	3.17±0.59	2.24±0.48
2	C17	1.40±0.40	1.23±0.34
3	7-MeC17	ND	0.79±0.33
4	3-MeC17	1.50±0.61	1.81±0.35
5	Octadecene*	1.50±0.61	ND
6	C18	6.89±1.49	5.59±1.10
7	7-MeC18	3.29±0.97	2.68±0.64
8	C19	3.31±0.75	3.20±0.82
9	9+11 MeC19	ND	2.29±1.34
10	3-MeC19	2.56±0.33	1.56±0.58
11	Eicosene*	ND	3.90±1.09
12	C20	8.96±1.37	7.26±1.40
13	C21	5.56±0.81	5.16±1.30
14	5-MeC21	2.81±1.13	ND
15	C22	12.43±2.08	10.06±2.06
16	C23	11.62±1.47	10.40±1.94
17	C24	9.53±1.04	9.38±1.83
18	C25	9.14±2.44	6.77±1.17
19	C26	3.78±0.37	3.11±0.75
20	C27	3.43±1.03	2.52±0.65
21	C28	5.25±1.12	6.82±1.36
22	C29	3.89±1.32	13.23±4.82

ND Not detected

* Double bond positions were not determined

There were 19 and 20 CHC compounds detected for the first instar larvae of *C. vicina* and *L. sericata* (respectively) (Table 4.4). In addition, the total percentage concentrations of n-alkanes and branched methyl alkanes detected were similar. However, the total concentrations of alkene compounds detected in the chemical profiles differed between the species. While the *C. vicina* first instar larvae had octadecene, *L. sericata* had eicosene in their CHC profiles.

Table 4.4: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for first instar larvae for each species reared outdoors

	<i>C. vicina</i>		<i>L. sericata</i>	
	number	Total % peak area	number	Total % peak area
n-alkanes	14	88	14	87
Branched methyl HC	4	10	5	9
Alkenes	1	1	1	4
<i>Total</i>	19	100	20	100

The chromatograms of the two species on the first day of the larval stage were similar, with higher concentrations of lower boiling point C20:H and C25:H compounds (Figure 4.4). Although the chromatograms look relatively similar, some key differences can be observed between the percentage concentrations of certain CHCs. The first instar larvae of both species consisted of the same n-alkane compounds, but C22:H and C23:H were the predominant n-alkanes in *C. vicina* larvae, while C23:H and C29:H were predominant n-alkanes in *L. sericata* larvae. Specifically, the percentage concentrations of C28:H and C29:H were significantly higher in *L. sericata* (13%) compared to in *C. vicina* (4%). In the profiles of both species, 3-MeC17 and 3-MeC19 were present, but at varying concentrations, with *C. vicina* yielding the highest concentrations. Branched methyl alkanes 7-MeC17 and 9+11-MeC19 were specific to the *L. sericata* species, while 5-MeC21 was specific for the *C. vicina*.

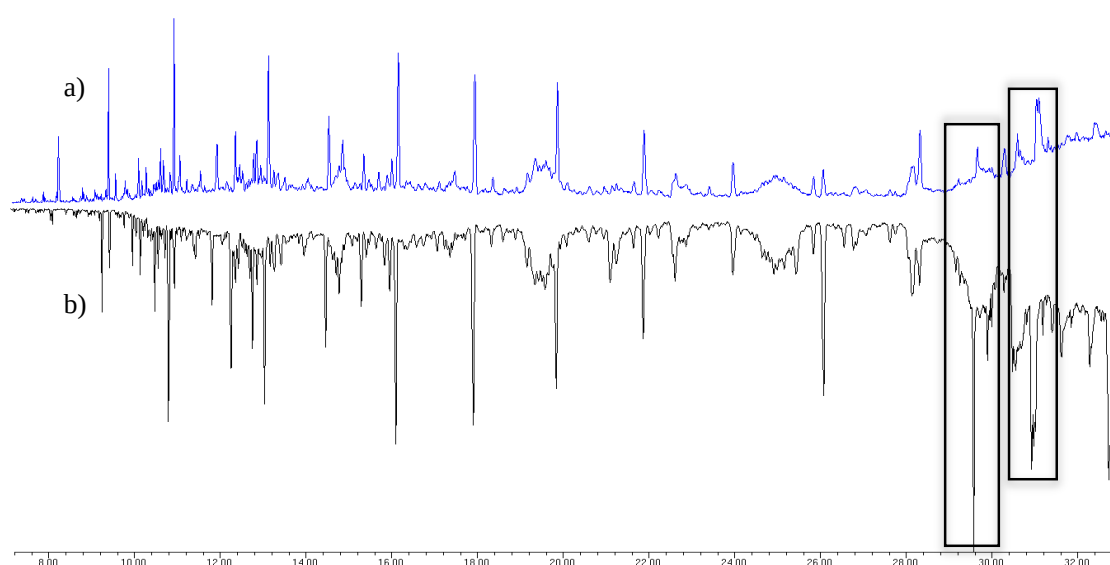


Figure 4.4: GC chromatograms showing CHC profiles of first instar larvae of blowfly species, a) *C. vicina*, b) *L. sericata* reared outdoors. The marked areas show the species-specific key regions within the CHC profiles

The presence/absence or different ratios of percentage concentrations in CHC profiles may be distinguished if there are not many samples for species identification, but this process becomes more complex as the number of species and samples increases. At this stage, applying statistical analysis to GC-MS data greatly simplifies the identification process. PCA was performed to identify the species using statistical analysis. In the interpretation of the PCA, the loadings for component pairs were shown as scatter plots. All CHC data given in Table 4.3 was applied for this purpose. PCA was performed using six principal components, describing 98.4% of the dataset variation within the first three components, comprising 72.2%, 24.5 and 1.7%, respectively. The PCA plot was created with the PC2 and PC3 scores.

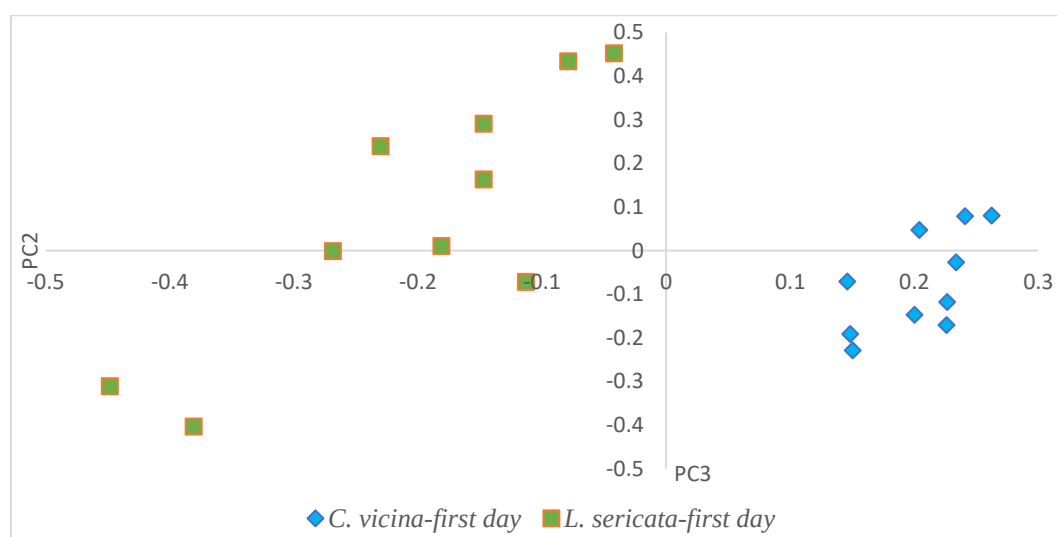


Figure 4.5: PCA plot showing PC3 against PC2 for *C. vicina* and *L. sericata* first instar larvae reared outdoors, using n-alkanes, alkenes and branched methyl alkanes

The PCA plot for the first instar larvae of *C. vicina* and *L. sericata* is given in Figure 4.5. The chromatograms extracted on the first day of the larval stages were very similar except for compound concentrations in quantitative analysis, but PCA provided separation data among the first instar larvae of both species based on quantitative analysis. Compounds that had large scores were docosane (C22:H), nonacosane (C29:H), 9+11-methylnonadecane and eicosene. In the plot, the cluster representing *C. vicina* first instar larvae is located on the right. In contrast, the cluster representing *L. sericata* is located on the left of the graph. As a result, the difference between the species is easily discernible.

4.3.2 Discussion of Larvae Identification

This study showed species identification between *C. vicina* and *L. sericata* first instar larvae reared outdoors using CHC analysis combined with GC-MS and PCA. It is imperative to quickly identify the first instar larvae with the CHC analysis, as it is challenging to identify the first instar larvae due to their relatively small size.

C. vicina and *L. sericata* larvae profiles contained the same number of CHCs. However, their concentrations were higher in *L. sericata* profiles than in *C. vicina*. Moore et al. [32] examined the differences in the CHC profiles of the first instar larvae of *L. sericata*, *C. vicina* and *C. vomitoria* species under $24\pm 1^{\circ}\text{C}$ temperature; the results obtained in the current study agreed with theirs. Furthermore, the stability of the CHC analysis technique under fluctuating temperatures makes it a suitable method for identifying the species of first instar larvae collected from the field. This means it is possible to identify the species by examining the CHC profiles, whether the larvae are collected from an indoor or outdoor crime scene. The presented results show that CHC analysis is a powerful tool to use in forensic entomology.

4.4 Puparial Stage

The insect cuticle composition is highly dynamic. When larvae form puparium, the cuticle plays multiple roles in regulating water loss, body temperature, intra or extra-colonial communication and defence against pathogens [136]. As pupae consist of the skin of third instar larvae, the cuticle of the puparia, as in larvae, can aid in identifying observable changes in the biochemical profile and its use in species differentiation and age estimation. Although pupae still carry their larval characters, some closely related species are indistinguishable even with an accurate morphological analysis of the puparia. Sclerotisation in the external profile of pupae makes it difficult to see morphological characters. Since pupae are generally similar in appearance, the pupae species are difficult to identify [67].

Although Amorim and Ribeiro [72] and Siri wattanarungsee et al. [71] identified the forensically important pupae species according to their morphological characters with SEM imaging, this complex method requires great taxonomic experience. Therefore, the most common method is to rear the pupae until the adult fly emergence; when the adult

flies subsequently emerge, their species can then be determined using published morphological keys. However, it takes time to rear the puparia.

Alternative methods have been developed over the years to identify pupae species, consisting of analysis of chemical profiles. Kranz et al. [55] extracted the blowfly pupae lipids and analysed them by total-vaporisation solid-phase microextraction (TV-SPME) GC-MS. They were able to identify four species according to concentration changes in their chemical profiles. Voss et al. [136] studied the determination of age and species of blowfly pupae using the hyperspectral imaging (HSI) technique and found a clear indication of discrimination accuracy of >92% among the species investigated during puparial development by linear discriminant classification modelling. HSI is a technique in which internal development can be followed without damaging puparial development. Analysis of the chemical profiles also reveals external developmental changes. Moore [40] provided a distinction between species by extracting the CHC profiles of *L. sericata* and *C. vicina* pupae reared under a constant temperature (24 ± 1 °C).

This study aimed to identify *L. sericata* and *C. vicina* using their CHC profiles extracted from the puparia, which was reared outdoors under fluctuating temperatures. For this purpose, the pupae were extracted on the first day of pupation and the last day just before they emerged as adult flies. The differences in CHC profiles were statistically determined, and species identification was carried out.

4.4.1 Results for Puparial Stage

The GC chromatograms of *C. vicina* and *L. sericata* pupae reared outdoors are given in Figures 4.6 and 4.7, and detected CHCs are shown in Table 4.5. The peak areas of CHCs with insufficient concentration (<0.5%) were not included in the analysis.

Table 4.5: CHC compounds extracted from *C. vicina* and *L. sericata* on the first and last days of the puparial stage reared outdoors, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>C. vicina</i>		<i>L. sericata</i>	
		First puparial day	Last puparial day	First puparial day	Last puparial day
1	C16	1.79±0.41	1.02±0.22	ND	ND
2	C17	ND	0.51±0.12	ND	ND
3	3-MeC17	1.40±0.18	0.99±0.13	ND	ND
4	Octadecene*	0.57±0.10	0.55±0.07	ND	ND
5	C18	3.07±0.37	2.96±0.33	ND	ND
6	3-MeC18	1.60±0.18	1.30±0.27	ND	ND
7	Eicosene*	ND	0.55±0.21	ND	0.66±0.06
8	C20	2.62±0.30	2.71±0.72	ND	0.50±0.08
9	3-MeC20	1.72±0.28	1.87±0.75	ND	ND
10	Docosene*	0.81±0.12	2.55±0.70	ND	0.50±0.03
11	C22	1.94±0.24	2.97±1.05	ND	ND
12	C23	ND	0.55±0.42	ND	ND
13	3-MeC23	1.14±0.14	1.33±0.30	ND	ND
14	C24	1.22±0.15	1.39±0.36	ND	ND
15	C25	49.95±8.94	33.89±7.35	7.38±1.50	5.50±0.60
16	11+13-MeC25	ND	0.50±0.16	ND	ND
17	3-MeC25	1.08±0.16	1.44±0.30	ND	ND
18	C26	2.43±1.09	2.06±0.50	ND	0.81±0.30
19	C27	10.22±2.52	15.05±4.72	20.20±4.85	23.24±6.74
20	11+13-MeC27	ND	0.89±0.20	ND	ND
21	3-MeC27	1.59±0.39	2.69±0.72	ND	1.41±0.71
22	C28	1.43±0.26	1.74±0.42	3.86±0.70	3.77±0.81
23	4-MeC28	ND	0.50±0.15	ND	ND
24	3-MeC28	ND	0.87±0.25	ND	ND
25	C29	6.31±1.31	7.93±1.75	48.92±8.48	46.01±8.29
26	7-Mec29	ND	0.50±0.12	0.58±0.23	0.57±0.20
27	5-MeC29	ND	0.50±0.12	ND	ND
28	3-MeC29	0.67±0.18	0.85±0.16	ND	ND
29	C30	0.61±0.24	0.63±0.15	2.36±0.49	2.25±0.52
30	8-MeC30	ND	0.56±0.27	ND	ND
31	4-MeC30	ND	0.50±0.11	ND	ND
32	C31	2.83±0.75	2.81±0.62	14.11±3.18	11.94±2.21
33	13+15-MeC31	1.63±0.45	1.34±0.42	ND	ND
34	3-MeC31	1.11±0.38	0.71±0.20	ND	ND
35	C32	1.17±0.44	1.33±0.36	1.46±0.40	1.37±0.38
36	C33	1.09±0.44	1.37±0.63	1.11±0.25	0.97±0.24

ND Not detected

* Double bond positions were not determined

Although *C. vicina* pupae had more n-alkane compounds in their profile, the total concentration of n-alkanes detected in *L. sericata* pupae was significantly greater than those of *C. vicina*. On the other hand, branched methyl alkanes detected in the chemical profile of *C. vicina* pupae were considerably more dominant than *L. sericata* in both number and total concentration; only one branched methyl alkane (7-MeC29) was found in *L. sericata*. While no alkenes were detected in the *L. sericata*, three were detected in the profiles of *C. vicina*.

Table 4.6: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for the first-day pupae for each species reared outdoors

	<i>C. vicina</i>		<i>L. sericata</i>	
	number	Total % peak area	number	Total % peak area
n-alkanes	14	87	8	98
Branched methyl HC	9	12	1	2
Alkenes	2	1	-	-
<i>Total</i>	25	100	9	100

When the chromatograms of the first day were examined (Figure 4.6), although the chromatograms appear to be similar, differences were evident in the percentage concentrations of CHC compounds. It was found that the profile of *C. vicina* pupae had CHCs with both short and longer chain lengths, whereas *L. sericata* pupae exhibited CHCs with a longer chain length. The dominant n-alkanes were C25:H, C27:H, C29:H and C31:H, which are all OLA and detected in the chemical profiles of both species. For *C. vicina* puparia, C25:H yielded the highest concentration, whereas C29:H was the dominant OLA in the profile of *L. sericata*. The percentage concentrations of C27:H, C29:H and C31:H in the chemical profiles of *L. sericata* pupae were higher than those of *C. vicina* puparia.

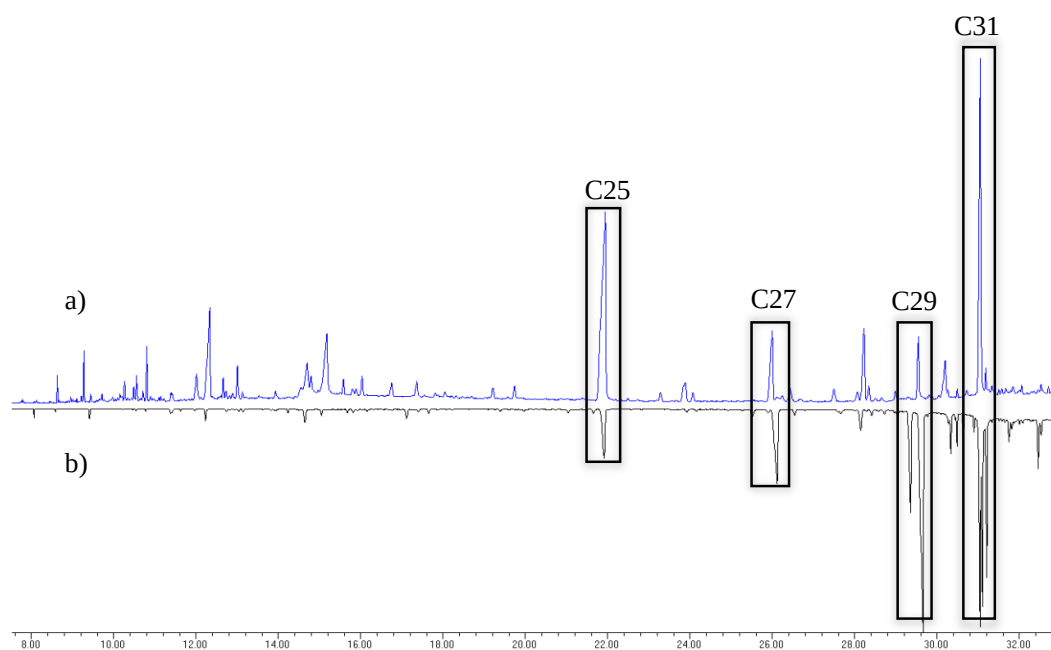


Figure 4.6: GC chromatograms showing the first day of puparial stages of the blowfly species, a) *C. vicina*, b) *L. sericata* reared outdoors. The marked areas show the species-specific key regions within the CHC profiles.

Chromatograms of the puparial profiles of *C. vicina* and *L. sericata* species on the last day of the puparial stage are given in Figure 4.7. When chromatograms were compared, as observed on the first day of the puparia, C25:H, C27:H, and C29:H n-alkanes yielded higher concentrations in *C. vicina* puparia, while C25:H, C27:H, C29:H, and C31:H were in higher concentration in *L. sericata* puparia. In addition, while C27:H, C29:H, and C31:H n-alkanes were higher in *L. sericata* puparia, the C25:H n-alkane had a significantly higher concentration in the chemical profile of *C. vicina* puparia.

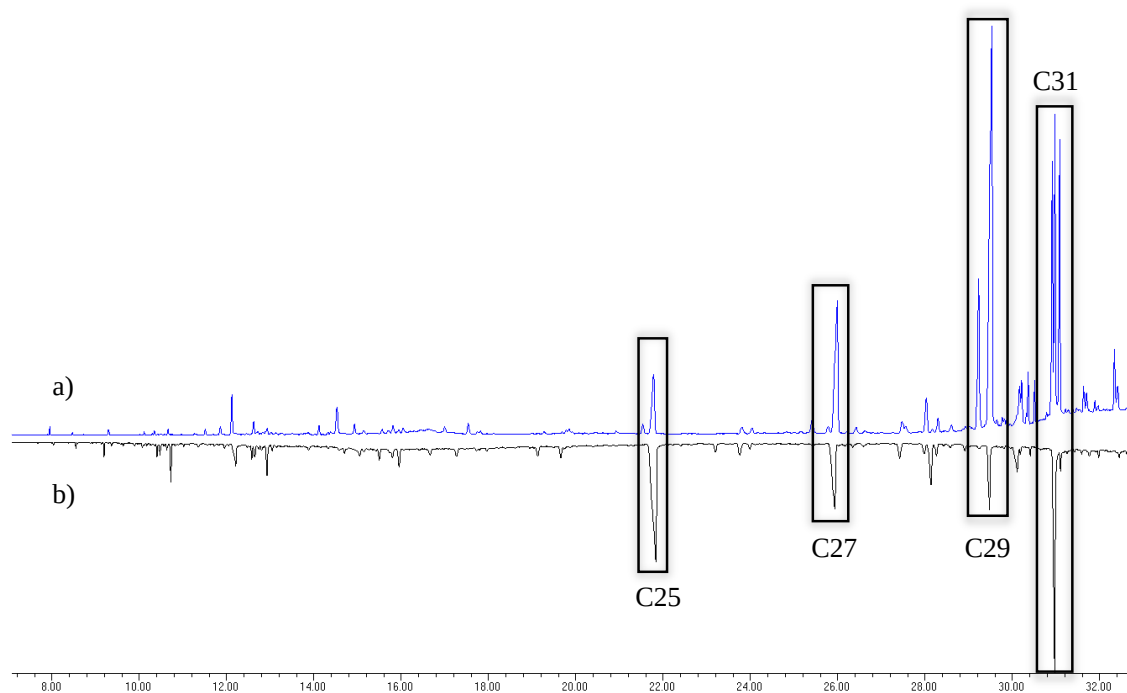


Figure 4.7: GC chromatograms showing the last day of puparial stages of the blowfly species, a) *L. sericata*, b) *C. vicina* reared outdoors. The marked areas show the species-specific key regions within the CHC profiles

The total numbers and total concentrations of n-alkanes, alkenes and branched methyl alkanes determined in the chemical profile of the pupae of *C. vicina* and *L. sericata* on the last day of the puparial stages are given in Table 4.7. The *C. vicina* pupae yielded more n-alkanes in comparison to *L. sericata*. However, the total n-alkane concentration detected in *L. sericata* pupae was higher than in *C. vicina*. The numbers and concentrations of branched methyl alkanes and alkenes detected in *C. vicina* were also higher than in *L. sericata*. In addition, 18-branched methyl alkanes were identified in *C. vicina* puparia. Only two of these compounds, 3-MeC27 and 7-MeC29, were detected in *L. sericata* pupae and their concentrations were lower than those of *C. vicina* puparia. Three alkene compounds were detected in *C. vicina* and two in *L. sericata*. The percentage concentration of alkenes detected in *C. vicina* pupae was higher than in *L. sericata*.

Table 4.7: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for the last day pupae for each species reared outdoors

	<i>C. vicina</i>		<i>L. sericata</i>	
	number	Total % peak area	number	Total % peak area
n-alkanes	14	75	10	91
Branched methyl HC	16	17	3	6
Alkenes	3	8	2	3
<i>Total</i>	33	100	15	100

When the chemical profiles of the two species were examined, some significant differences were observed. For this reason, statistical analysis was applied to identify the species. First, PCA was performed to identify the pupae species statistically. All CHC data given in Table 4.5 were used for PCA, which was performed using six principal components, describing 99.8% of the dataset variation, with the first three components comprising 69.5, 29.6 and 0.7, respectively. Figure 4.8 was created using the PC2 and PC3 scores. Compounds that exhibited the highest score values and differentiated between species were pentacosane (C25:H), heptacosane (C27:H) and nonacosane (C29:H).

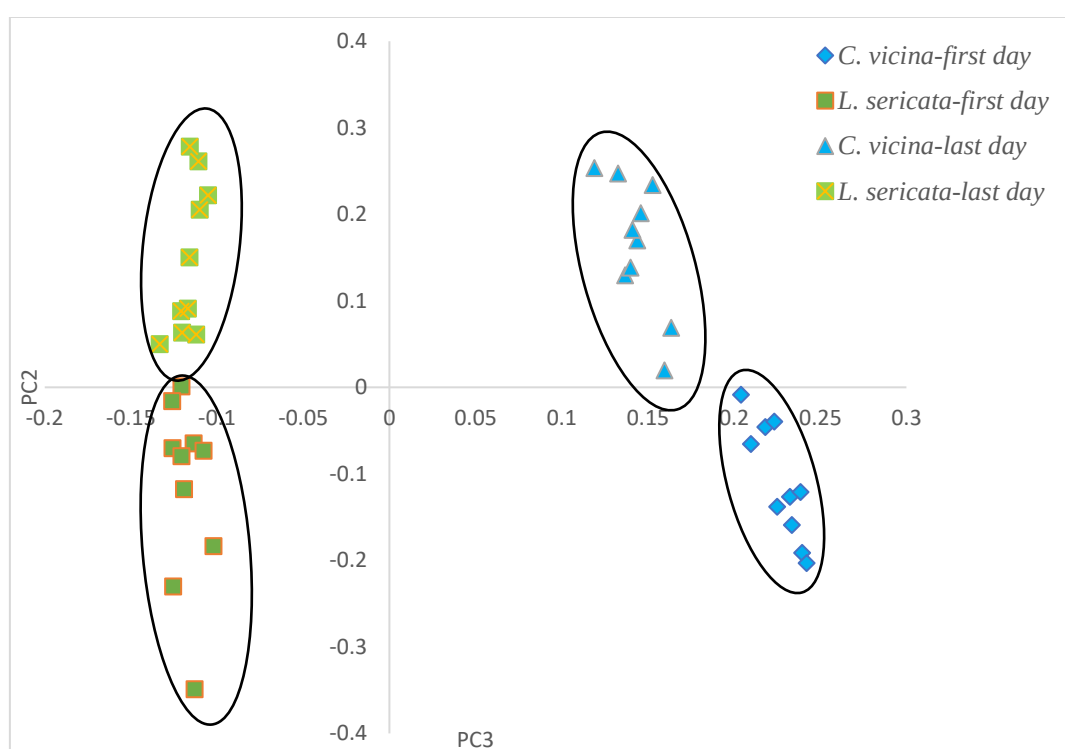


Figure 4.8: PCA plot showing PC3 against PC2 for *C. vicina* and *L. sericata* pupae reared outdoors, using n-alkanes, alkenes and branched methyl alkanes

As shown on the PCA plot, clusters representing pupae of *C. vicina* were located in the positive area of PC2 loading and clusters representing *L. sericata* pupae were located in the negative area. With the application of PCA to the GC-MS data, a distinction between species was obtained in the pupae of *C. vicina* and *L. sericata* both on the first day and

last day of the pupae before the adult fly stage. In addition, clusters differed based on age; the ageing of pupae is discussed in Chapter 5.

4.4.2 Discussion of Pupae Identification

GC-MS analysis was used to analyse the extracted CHC profiles of pupae species and to group them statistically with PCA. *C. vicina* had short and long-chain length CHCs in their chemical profiles; the chemical profile of *L. sericata* had CHCs with longer chain lengths. The predominant group in the pupae was n-alkanes, and very few branched methyl alkanes and alkenes were detected. Pupae profiles had significantly fewer CHCs compared to the larval stage. There was significantly fewer branched methyl, and the profiles contained n-alkanes with longer carbon chains. Branched methyl alkanes and alkenes detected have a lower boiling point. Pupae is a hard protective layer, which may explain why a simple profile consisting mainly of straight-chain n-alkanes with higher boiling points was observed. Kranz et al. [55] distinguished *Cochliomyia macellaria*, *L. cuprina*, *L. sericata*, and *P. regina* pupae species only based on pentadecane rather than the entire chemical profile. The results presented in this study also support that other OLAs, such as pentacosane and nonacosane, can be sufficient to distinguish between species.

Overall, the results detected in this section were in line with those presented by Moore [40], who conducted the experiments under constant temperatures. In this study, pupae were reared in the field and exposed to fluctuating temperatures. With the understanding that some differences in the CHC profiles remain constant, regardless of the temperature factor, this section provides enough information to distinguish between the two presented species using PCA.

4.5 Empty Puparia

Empty puparia can be found near human remains even after the course of years and can be used to estimate PMI_{min} since their chemical degradation is slow. Mostly, morphological characters, the anterior end (second to fourth segments) and posterior spiracle are used for pupae identification [137]. In contrast, identification of empty puparia is more difficult as adult flies break the pupae when they emerge. Researchers

have explored empty puparia and developed new identification methods, including CHC analysis, which has some significant advantages [78]:

- A small piece of empty puparia is sufficient to be able to extract the chemical profile.
- CHCs are relatively stable molecules; they can be used for ancient samples.
- Pinned museum samples can be analysed with this method.
- A single sample can provide a functional GC-MS template.
- Compared to other identification methods, such as DNA analysis, it is a simple, applicable and inexpensive technique.

Ye et al. [78] examined the CHC profiles of the empty puparia (exuviae) from six necrophagous species and pioneered the use of empty puparia to identify species in forensic entomology. Moore [40] achieved species identification for empty puparia of *C. vomitoria*, *C. vicina* and *L. sericata* using CHC analysis, showing that CHC characters of empty puparia could be classified chemotaxonomically. However, the aforementioned studies were conducted on samples reared under constant temperature in controlled laboratory conditions. Therefore, the effects of environmental conditions on the stability of CHCs and the use of CHC analysis in the identification of pupae and empty puparia species are unknown.

This study, considering the advantages of the CHC analysis, aimed to identify the species by extracting the chemical profiles of the remaining empty puparia after the emergence of adult flies from *C. vicina* and *L. sericata* pupae (Day 1). The empty puparia were obtained from the pupae reared outdoors during the experimental period.

4.5.1 Results for Empty Puparia

The empty puparia left behind by adult flies on the first day of emergence were collected and examined to identify *C. vicina* and *L. sericata* species. The CHCs detected in the extractions of the CHC profiles are given in Table 4.8, with the total percentage of each compound present and the percentage standard deviation. The peak areas of CHCs with insufficient concentration (<0.5%) were not included.

Table 4.8: CHC compounds extracted on Day 1 of *C. vicina* and *L. sericata* empty puparia reared outdoors, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>C. vicina</i> Day 1	<i>L. sericata</i> Day 1
1	C16	0,77±0,46	nd
2	C18	1,27±0,82	nd
3	C19	0,39±0,20	nd
4	4-MeC19	0,57±0,34	nd
5	3-MeC19	0,63±0,22	nd
6	C20	1,24±0,69	nd
7	C21	0,25±0,10	nd
8	C22	1,05±0,51	nd
9	C23	0,81±0,51	nd
10	C24	0,62±0,34	nd
11	C25	22,64±5,78	4,71±0,87
12	11+13 MeC25	0,67±0,33	nd
13	3-MeC25	1,60±0,91	nd
14	C26	2,27±1,41	1,20±0,19
15	13+15 MeC26	1,11±0,51	nd
16	3-MeC26	1,16±0,63	nd
17	C27	25,11±13,33	26,78±4,22
18	13+15 MeC27	1,05±0,75	nd
19	11+13 MeC27	2,96±1,55	nd
20	7-MeC27	1,17±0,55	nd
21	5-MeC27	0,73±0,34	nd
22	4-MeC27	0,64±0,28	nd
23	3-MeC27	6,81±3,15	4,05±0,89
24	C28	2,12±0,89	3,32±0,67
25	13+15 MeC28	1,65±0,82	0,71±0,18
26	11+13 MeC28	0,54±0,28	nd
27	4-MeC28	1,77±0,88	nd
28	3-MeC28	nd	1,10±0,21
29	C29	9,54±2,99	33,17±5,67
30	13+15 MeC29	0,63±0,33	4,62±0,56
31	11+13 MeC29	2,48±1,05	nd
32	7-MeC29	0,70±0,30	0,90±0,37
33	4-MeC29	nd	1,48±0,52
34	3-MeC29	0,82±0,39	2,69±0,90
35	C30	0,36±0,12	1,40±0,49
36	8-MeC30	0,55±0,21	0,68±0,34
37	3-MeC30	0,41±0,13	1,09±0,27
38	C31	1,97±0,65	9,06±1,84
39	13+15 MeC31	nd	1,51±0,52
40	3-MeC31	nd	0,79±0,28
41	C33	0,59±0,20	0,74±0,21

ND Not detected

The total numbers and percentage concentrations of n-alkanes and branched methyl alkanes detected in the chemical profiles of *C. vicina* and *L. sericata* empty puparia are given in Table 4.9. In *C. vicina* chemical profiles, more n-alkane compounds were detected, but the total concentration of n-alkanes detected in *L. sericata* profiles was higher than in *C. vicina*. Thus, although the number of branched methyl alkanes was more significant than the n-alkanes, the total n-alkane concentration was three times greater than the branched methyl alkane concentration. While 21 branched methyl alkane compounds were detected in *C. vicina* empty puparia, this number was 11 in *L. sericata* profiles. The total concentration of branched methyl alkanes detected in *C. vicina* was higher than that of *L. sericata*. Whereas the branched methyl alkanes detected in *C. vicina* profiles had from C19:H to C30:H carbon numbers, those detected in *L. sericata* had from C28:H to C31:H carbon numbers. The predominant branch methyl alkane in *C. vicina* profiles was 3-MeC27. Other higher concentration ones were 11+13-MeC27 and 11+13-MeC29. In contrast, the predominant branch methyl n-alkane in *L. sericata* profiles was 13+15-MeC29. Other higher concentration compounds were 3-MeC27 and 3-MeC29.

Table 4.9: CHC composition – numbers and percentage concentrations of n-alkanes and branched methyl alkanes for Day 1 empty puparia for each species stored outdoors

	<i>C. vicina</i>		<i>L. sericata</i>	
	number	Total % peak area	number	Total % peak area
n-alkanes	16	71	8	80
Branched methyl HC	21	29	11	20
Alkenes	-	-	-	-
<i>Total</i>	37	100	19	100

GC chromatograms of the empty puparia of *C. vicina* and *L. sericata* on Day 1 are given in Figure 4.9. In *C. vicina* empty puparia, CHCs with C25:H to C33:H carbon numbers were detected densely. Furthermore, CHCs from C16:H to C24:H carbon numbers were detected even though they were in lower concentrations (>0.5%). In contrast, in *L. sericata* empty puparia, only CHCs with C25:H to C33:H carbon numbers were detected. In *C. vicina* empty puparia, the percentage concentration of C25:H was approximately five times higher than in *L. sericata*. In *L. sericata* empty puparia, the C29:H concentration was approximately three times, and the C31:H concentration was approximately five times higher than that of *C. vicina*. The C27:H concentration was almost similar in both species. Thus, C25:H and C27:H compounds were abundant in *C. vicina* profiles, while C29:H and C31:H compounds were abundant in *L. sericata* profiles.

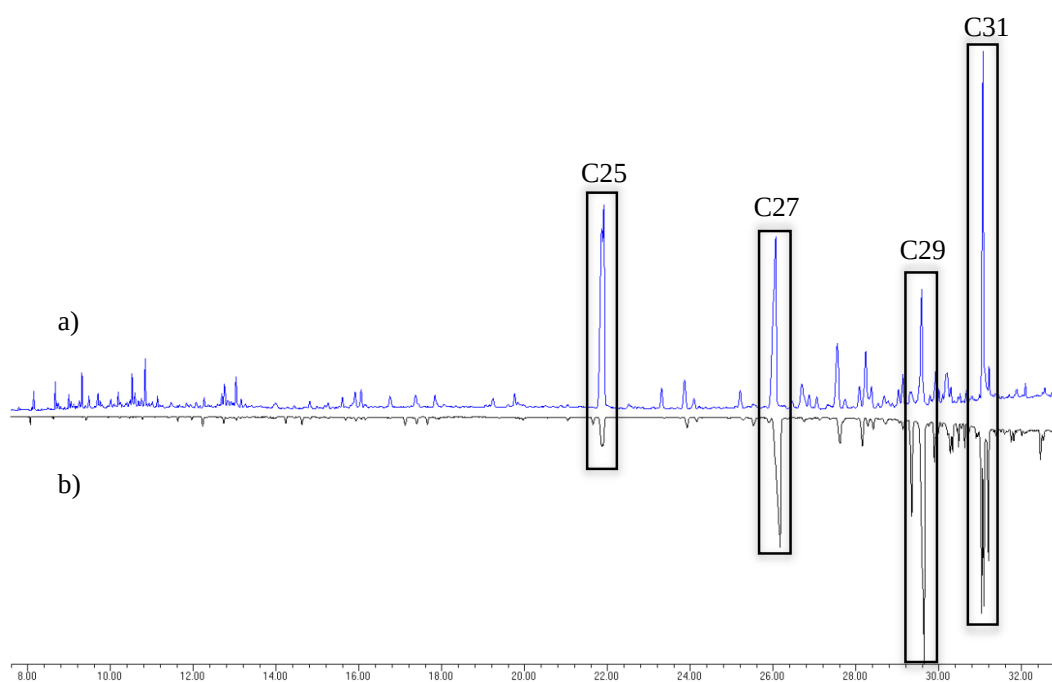


Figure 4.9: GC chromatograms showing Day 1 of empty puparia of the blowfly species, a) *C. vicina*, b) *L. sericata* stored outdoors. The marked areas show the species-specific key regions within the CHC profiles

Although differences in species profiles were evident in their chromatograms by looking at qualitative and quantitative differences in the CHC profile of empty puparia, PCA demonstrated the species identification. PCA was performed using six principal components, describing 99.6% of the dataset variation, with the first three components comprising 82.8, 15.7 and 1.2%, respectively. Figure 4.10 was formed with the PC2 and PC3 scores. Compounds that give the highest scores in PCA calculations and provide differentiation between species were pentacosane (C25:H), heptacosane (C27:H) and nonacosane (C29:H).

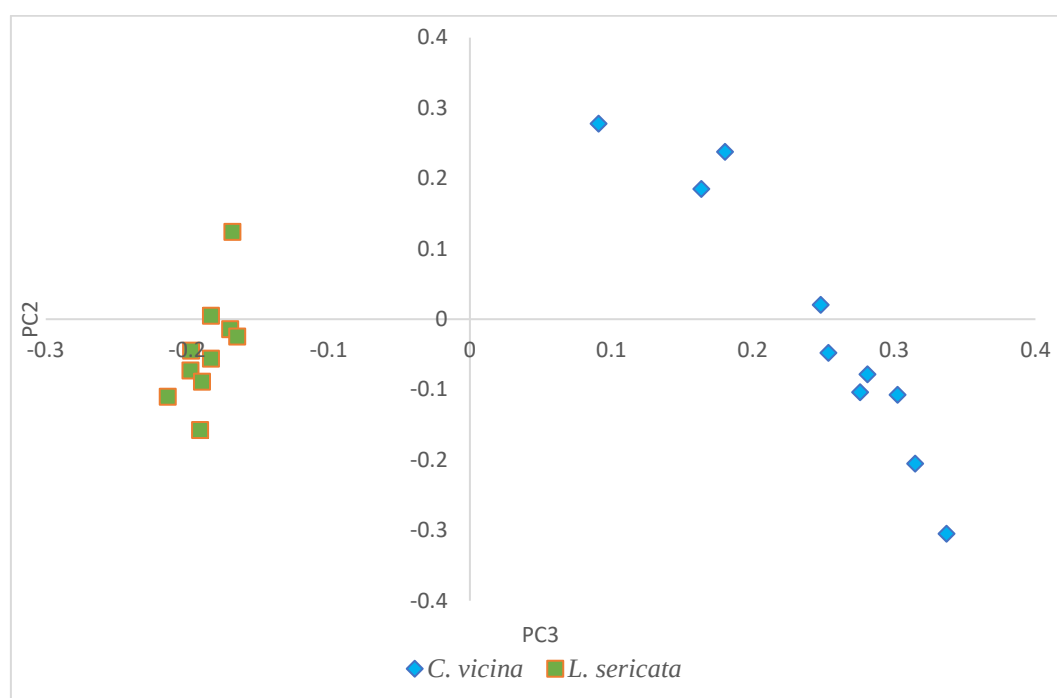


Figure 4.10: PCA plot showing PC3 against PC2 for *C. vicina* and *L. sericata* empty puparia stored outdoors, using n-alkanes and branched methyl alkanes

All the CHC compounds shown in Table 4.8 were applied to PCA to differentiate between *C. vicina* and *L. sericata* empty puparia. A clear distinction was obtained between the species. As seen in the plot, clusters representing the empty puparia of *C. vicina* were in the positive area of the PC2 loading, while the clusters representing the empty puparia of *L. sericata* were in the negative area.

4.5.2 Discussion of Empty Puparia Identification

The results presented in this section provide species identification of empty puparia of *C. vicina* and *L. sericata* stored outdoors. GC-MS and PCA techniques of CHC analysis were demonstrated to be reliable tools for species identification of empty puparia. A comparison of empty puparia and pupae chemical profiles revealed that more CHCs were detected, especially more branched methyl alkanes in CHC profiles of empty puparia. This is because pupae extractions include only CHCs extracted from the outer cuticle surface of the puparia. On the other hand, empty puparia extractions contain the CHC profile of the pupae and traces of the CHC profiles left behind by adult flies. This similarity provides a link between adult fly species and their empty puparia.

Braga et al. [138] obtained chemotaxonomic species differentiation by analysing the chemical profiles of four species of Sarcophagidae empty puparia with the CHC analysis. However, they stored the insects in similar conditions in the laboratory and stated that it is unknown whether similar results can be obtained with adult flies collected directly from the field. Therefore, the current study is aimed to improve the CHC analysis by conducting field studies.

Musah et al. [139] analysed CHCs profiles from empty puparia of *Chrysomya rufifacies*, *L. sericata*, *L. cuprina* and *C. macellaria* using direct analysis in real-time-mass spectrometry (DART-MS). All species on the PCA plot were clearly separated from one another, demonstrating that CHC profiles have unique chemical differences. However, there was less differentiation between *L. cuprina* and *L. sericata*. Compared to other species, their profiles exhibit more resemblance because they belong to the same genus (*Lucilia*). Nevertheless, CHC analysis is advantageous because, despite weathering effects, chemical information may be maintained in both young and old empty puparia due to the stability of the CHCs.

Studies conducted outdoors showed that *C. vicina* contained many CHCs in its chemical profile, while *L. sericata* had a more straightforward chemical profile. From this point of view, even if the temperature fluctuates, the chemical profile between the species remains constant and enables species identification. It is of great importance in a forensic investigation that empty puparia remain at a crime scene for a long time. The species identification based on CHC composition indicates that this is a very reliable technique in insect taxonomy and plays an essential role in chemotaxonomy.

4.6 Overall Discussion of Species Identification

The primary purpose of this study was to demonstrate the usability of CHC profiles as a species-specific separator for field samples. Since larval growth rates vary greatly according to species, species identification is necessary before applying ageing methods to samples. When taxonomic identification of the insect is impossible based on morphology or its DNA is severely damaged (e.g., empty puparia contain relatively little DNA, which degrades over the age if exposed to the environment) [138], the CHC analysis technique can be employed as an alternative; it also dramatically increases time efficiency compared to entomological methods.

The CHC profiles of *C. vicina* and *L. sericata* species eggs, larvae, pupae and empty puparia were obtained using a simple extraction technique. Examination of chemical profiles with GC-MS and statistical analysis with PCA results showed that the CHC profile of each species is unique. Roux et al. [135] showed that *C. vomitoria*, *C. vicina* and *P. terraenovae* blowflies differ between species according to their CHC profiles in all life cycles. Moore [40] also conducted research using the CHC analysis for *C. vomitoria*, *C. vicina* and *L. sericata* blowfly species. Both studies were carried out under constant temperature in laboratory conditions. In contrast, the current research collected species from the wild and reared them outdoor under fluctuating temperatures. Nevertheless, the results obtained in the life stages were consistent with previous studies. Although temperature has an undeniable effect on CHC profiles [89], this study showed that it is still possible to identify a species based on the differences in CHC profiles between species. Furthermore, the effects of temperature and geographical location on CHC profiles are examined in detail in later chapters.

Species identification was achieved in this study by CHC analysis in egg and puparial stages, where key morphological characters are rare. However, the results presented are preliminary. The content of this study is limited to the two most common and forensically important blowfly species. Further research involving more forensically important adult fly species is needed to establish a CHC database across species. Larval results are valuable as they provide an easy procedure, especially for first instar larvae, where identification requires more experience and time due to their small size. Also, species identification can be obtained in a short time thanks to CHC analysis and PCA. The *C. vicina* and *L. sericata* species selected in this study are from the same family, but their

adult flies can be easily distinguished according to their colour and size. For this reason, species identification in the adult fly stage was excluded from the scope of this chapter.

Nevertheless, this technique shows great potential for species identification in forensic entomology by allowing accurate, cheap and fast taxonomical information regardless of location and temperature.

Chapter 5

Age Estimation Using Cuticular Hydrocarbons

5.1 Introduction

PMI_{min} is crucial in murder investigations as it is critical to know the time of death in order to establish and evaluate the series and course of events in criminal cases [26][140]. In forensic entomology, species identification and age estimation of Calliphoridae species have been widely studied to establish the PMI_{min} [42]. When the identification has been established, the next step is to estimate the age of the insect evidence by one of two methods. The first is comparing larval sizes with values given in isomegalen and isomorphen diagrams in proportion to temperature and time [47]. This method has some disadvantages, including that a lack of food or competition between species might result in larval mortality or a drop in larval growth and weight [141]. In an indoor environment where the temperature remains almost constant, the age of larvae can be estimated from the larval length, but because outdoor temperatures fluctuate significantly, only a broad age range can be estimated with this method. Also, this method is only applicable for larval stages [15], and there is a limited number of published references using isomegalen and isomorphen diagrams.

Another method is Accumulated Degree Days or Hours (ADD/H). In general, the ADD/H formula is species-specific, so the minimum growth threshold should be well known. However, PMI_{min} is difficult to determine in this way, as decomposition does not occur in response to a single temperature threshold [142]. Based on previous studies, the ADD/H values vary, and key threshold values required for other species are scarce. All of these circumstances have highlighted the necessity to research novel insect-based

PMI_{min} estimate techniques. Techniques such as CHC analysis are currently considered an outstanding approach, as they have the potential to provide a more accurate and quicker result for colonization time estimates. Studies on Diptera [24,42–45,143] have shown that CHCs can be used for age estimation as well as taxonomic purposes.

Trabalon et al. [144] performed the first study to correlate the change in the CHC profile of *Calliphora vomitoria* adult flies with age and gender. They determined intergroup differences using discriminate analysis based on the relative ratios of male and female CHC profiles. This study also showed that young adult flies could be distinguished from older ones, and males can be distinguished from females through the chemical profile. Roux et al. [135,145] presented species identification and age-related differences in developmental life stages among *C. vicina*, *C. vomitoria* and *Protophormia terraenovae* using CHC analysis. Moore et al. [24] showed that the CHC analysis of *L. sericata* using GC-MS with PCA produced different datasets for different larval ages in which each cluster was linked to a specific age. The larvae were reared at a constant 24°C, and the analysis was completed for the larvae stage. Xu et al. [77] characterised the age-dependent, quantitative changes in the CHC profile of *Aldrichina grahami* larvae using GC-MS.

Most experiments on developmental patterns of forensically important blowflies aim to collect data under constant temperatures, and fluctuating temperatures have scarcely been mentioned in most studies [146]. However, in the majority of forensic investigations, the temperature at the crime scene is constantly fluctuating. This chapter examines the effects of fluctuating outdoor temperature and indoor constant temperature on chemical profiles in relation to age estimations performed using CHC analysis in the life stages of *C. vicina* and *L. sericata* species. The experimental setup consisted of a fieldwork group reared from egg to adult fly in a cage placed in a field and a control group reared in a constant temperature (25±1°C) incubator. The CHC profiles were analysed using GC-MS, and PCA was applied to visualise the results and obtain clusters based on age.

5.2 Larval Stage

Age estimation of the immature stages of these necrophagous is an important step for estimating colonisation time and determining PMI_{min} [80]. In the larval age estimation, generally, the body length, width and weight of the developing larvae are measured, and

the time required to reach that stage is estimated [147]. The time required to reach the different developmental stages is affected by temperature. While this period is shorter at higher temperatures, it takes longer at lower temperatures. Therefore, temperature data at the crime scene must be measured as well as taken from the nearest meteorological station. However, when calculating the development rates, it should be considered that the temperature data taken from the station are not the same as the temperatures experienced by the insects on the corpse based on the metabolic and feeding rate of larvae [16].

VanLaerhoven [140] mentioned the need for validation studies of PMI_{min} estimates based on entomological evidence. According to their studies, four entomologists, each using slightly different methods during the Westerfield trial, gave PMI_{min} estimates that either overlapped or came within four days of overlapping based on these methods. The development rates and biology of larvae also vary according to the larvae species. For example, the larvae of *L. sericata* develop faster at 25°C than *C. vicina* larvae. [16]. Therefore, larvae found on a corpse at the same stage of development do not indicate that they are the same age or have the same colonisation time. The food source is another factor that affects the development of the larvae; if the larvae cannot find an adequate food source to feed on, their development slows down. Accordingly, it is seen that it is not possible to determine precisely whether these estimates are objectively accurate without knowing the error rate of different PMI_{min} estimates using different approaches.

CHC analysis shows the potential to be a new tool against the limitations of physiological methods in estimating larval age. Previous studies have shown that many other insect species have age-related differences in CHC profile. Studies on *Chrysomya rufifacies* [22], *A. grahmi* [77], *C. vomitoria*, *C. vicina*, *P. terraenovae* and *L. sericata* [24,32,42,145,148] give examples of age-related quantitative changes in CHC profiles of Diptera species.

In previous studies, the developed models often used constant temperature cycles and laboratory environments for the rearing process of the samples. However, the research presented in this thesis examined *L. sericata* and *C. vicina* larvae exposed to natural environmental conditions in the UK. A control group was also examined by rearing a separate experiment set at 25±1°C constant temperature in the laboratory. A reliable method of age estimation should be able to accurately estimate the age of samples reared

in the field as well as species reared in controlled laboratory conditions. The data in this part of the chapter are based on the entire larval stage of *C. vicina* and *L. sericata*. Results from GC-MS analysis and subsequent PCA results will be discussed in detail for each species.

5.2.1 Results for Larvae

5.2.1.1 *C. vicina* larvae

5.2.1.1.1 Fieldwork study

A total of 36 CHC compounds identified by GC-MS chromatograms of *C. vicina* reared outdoors are given in Table 5.1. The total CHC concentrations of CHC profiles were n-alkanes (51%), alkenes (6%) and branched methyl alkanes (43%) from Days 1 to 11. The chain lengths ranged from C16:H to C33:H. The positions of the double bonds in alkenes were not determined. The predominant group in concentration and number was n-alkanes in the chemical profiles.

Table 5.1: CHC compounds extracted from *C. vicina* larvae reared outdoors, total percentage of each compound present and percentage standard deviation for each day

Peak No	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	DAY 10	DAY 11
1	C16	3.16±0.59	2.47±1.08	2.41±0.64	1.97±0.52	tr	2.04±0.47	2.71±0.63	1.02±0.37	1.29±0.52	1.70±0.57	1.78±0.70
2	C17	1.39±0.40	1.09±0.50	1.20±0.38	1.10±0.33	tr	1.26±0.30	1.58±0.38	tr	0.95±0.27	1.42±0.30	1.26±0.50
3	7-MeC17	tr	tr	0.77±0.25	0.81±0.20	tr	0.85±0.25	1.01±0.29	tr	tr	1.07±0.26	0.96±0.39
4	3-MeC17	1.81±0.48	1.34±0.40	1.37±0.37	1.28±0.32	tr	1.54±0.31	1.63±0.46	0.99±0.47	1.37±0.42	2.35±0.57	1.99±0.62
5	Octadecene*	1.49±0.61	1.95±1.80	1.51±0.57	1.76±0.51	tr	1.90±0.48	1.65±0.49	1.08±0.53	1.25±0.48	2.09±0.55	2.25±0.77
6	C18	6.87±1.48	5.35±1.78	5.53±1.31	4.33±0.86	0.52±0.16	4.49±0.90	5.78±1.41	3.36±1.22	4.06±1.28	5.33±1.18	4.99±1.36
7	7-MeC18	3.28±0.97	2.47±0.82	2.71±0.81	2.52±0.72	tr	2.64±1.12	2.73±1.11	1.68±0.83	1.89±0.75	2.81±0.69	2.60±0.83
8	C19	3.30±0.75	2.70±0.92	2.34±0.56	2.17±0.67	0.75±0.22	2.23±0.75	2.58±0.72	1.99±0.50	2.06±0.62	2.69±0.68	2.13±0.76
9	9+11-MeC19	tr	tr	1.81±0.51	3.78±7.62	0.66±0.16	6.65±8.81	1.33±0.39	4.67±10.98	1.65±0.23	1.96±0.34	3.74±6.73
10	3-MeC19	2.55±0.33	1.91±0.63	1.55±0.30	1.37±0.48	0.65±0.14	1.34±0.36	1.60±0.50	1.29±0.30	1.50±0.30	1.62±0.30	1.42±0.31
11	C20	8.93±1.37	7.63±1.77	6.10±1.11	4.48±0.93	2.36±0.51	4.64±1.04	5.04±1.12	5.05±1.23	5.36±1.11	5.73±0.79	5.23±0.99
12	3-MeC20	tr	1.92±0.58	1.58±0.83	1.14±0.32	tr	1.39±0.40	tr	tr	tr	tr	tr
13	C21	5.54±0.80	4.99±1.08	3.31±0.84	2.59±0.47	2.18±0.52	2.72±0.74	2.95±0.56	3.26±0.81	3.12±0.61	3.28±0.51	2.84±0.58
14	5-MeC21	tr	2.13±0.82	1.77±0.57	1.35±0.27	tr	1.65±0.51	tr	tr	tr	tr	tr
15	4-MeC21	2.80±1.13	1.68±0.31	1.35±0.41	1.18±0.39	tr	tr	1.52±0.73	1.21±0.76	tr	1.46±0.52	1.48±0.61
16	3-MeC21	tr	tr	1.57±0.35	1.22±0.47	1.94±0.73	tr	tr	tr	tr	3.12±1.96	1.58±0.60
17	Docosene*	tr	tr	tr	tr	tr	2.44±0.61	2.65±0.29	2.48±0.80	3.33±0.49	4.14±0.74	3.88±0.77
18	C22	12.39±2.07	10.85±2.07	8.03±1.20	5.32±1.07	5.12±0.99	5.53±1.17	6.27±1.04	6.87±1.48	6.86±1.06	7.37±1.06	6.34±1.19
19	C23	11.58±1.47	10.31±2.03	7.46±1.11	4.99±1.05	5.13±0.96	4.43±0.80	5.64±1.05	6.66±1.74	6.17±0.99	6.03±0.86	5.29±0.93
20	5-MeC23	tr	3.03±0.68	tr	tr	tr	1.37±0.48	tr	tr	tr	1.64±0.46	1.96±0.72
21	3-MeC23	tr	5.89±2.15	tr	tr	tr	tr	tr	tr	tr	tr	tr

Peak No	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9	DAY 10	DAY 11
22	C24	9.50±1.04	9.89±1.99	5.96±0.78	4.13±1.24	5.25±1.01	3.56±0.59	4.17±0.77	4.93±1.03	4.46±0.60	4.26±0.69	4.58±1.64
23	C25	9.11±2.43	6.24±0.98	7.78±3.01	6.31±1.61	5.42±0.98	3.42±0.67	4.07±0.75	4.32±1.52	3.91±0.70	3.69±0.51	3.60±0.69
24	11+13-MeC25	tr	tr	2.19±0.60	tr	tr	tr	tr	tr	2.09±0.43	tr	tr
25	C26	3.77±0.37	3.28±0.55	3.54±1.28	2.32±0.46	2.09±0.33	2.63±1.02	1.98±0.27	2.19±0.51	2.28±0.47	2.25±0.26	2.19±0.39
26	C27	3.42±1.03	2.35±0.51	6.50±2.56	10.24±2.34	10.91±4.51	3.59±0.62	5.77±1.28	7.74±2.62	7.22±2.25	5.60±1.00	9.71±3.18
27	3-MeC27	tr	tr	2.48±0.71	2.95±0.66	3.50±0.83	tr	1.46±0.42	1.15±0.32	1.21±0.27	1.56±1.30	1.14±0.28
28	C28	5.23±1.12	5.23±1.24	4.02±0.72	3.68±1.21	3.48±1.31	3.40±0.51	3.78±1.08	4.73±1.59	6.02±1.39	5.35±1.40	5.40±1.45
29	C29	3.88±1.32	2.66±1.35	7.18±2.98	11.65±2.52	17.36±4.58	11.98±2.56	15.41±2.46	21.74±5.58	20.03±5.98	12.43±3.40	13.05±3.88
31	3-MeC29	tr	tr	1.42±1.04	1.12±0.28	2.36±0.61	0.62±0.21	0.88±0.29	0.94±0.22	0.94±0.31	0.79±0.49	0.60±0.13
32	C30	tr	2.63±1.67	2.01±0.49	2.59±0.92	2.92±0.77	2.07±0.39	2.06±0.59	2.60±1.35	2.93±1.06	2.78±0.68	2.88±1.13
33	3-MeC30	tr	tr	tr	tr	tr	8.61±14.37	5.77±14.61	tr	tr	tr	tr
34	C31	tr	tr	4.54±2.88	8.97±1.71	15.67±3.37	7.22±0.97	5.07±0.92	4.88±1.78	4.70±1.99	2.94±0.87	2.61±0.62
35	C32	tr	tr	tr	tr	5.38±2.44	1.19±0.37	1.19±0.34	1.63±0.45	1.75±0.55	1.11±0.38	1.19±0.33
36	C33	tr	tr	tr	2.69±0.65	6.34±1.50	2.58±0.28	1.71±0.35	1.56±0.68	1.60±0.78	1.43±0.63	1.35±0.34

tr = Trace amount detected (<0.5%)

* Double bond positions were not determined

Figure 5.1 shows the chromatograms of the larval developmental stage of *C. vicina* reared outdoors for Days 1, 2, 4, 7, 9 and 11. Sometimes a decrease or increase in concentrations and the presence/absence of a compound in a chromatogram can serve as a specific age indicator for that age. Examining changes in chromatograms over age and making comparisons can reveal how chemical profiles change with age. As the retention time increases in the chromatograms, CHCs with higher boiling points and longer carbon chains are detected. When the CHC profiles of the larvae were examined, it was discovered that long-chain CHCs such as C30:H to C33:H were not detected in the profiles of the Days 1, 2 and 3 larvae, which were known to be in the immature stage, but that these CHCs began to be detected in the profiles of the mature stage larvae. As the larvae continued to age and develop, the shorter-chain CHCs that were more prevalent in Day 1 larvae, which provided a more flexible structure to the larvae, began to be replaced by longer chain and branched CHCs.

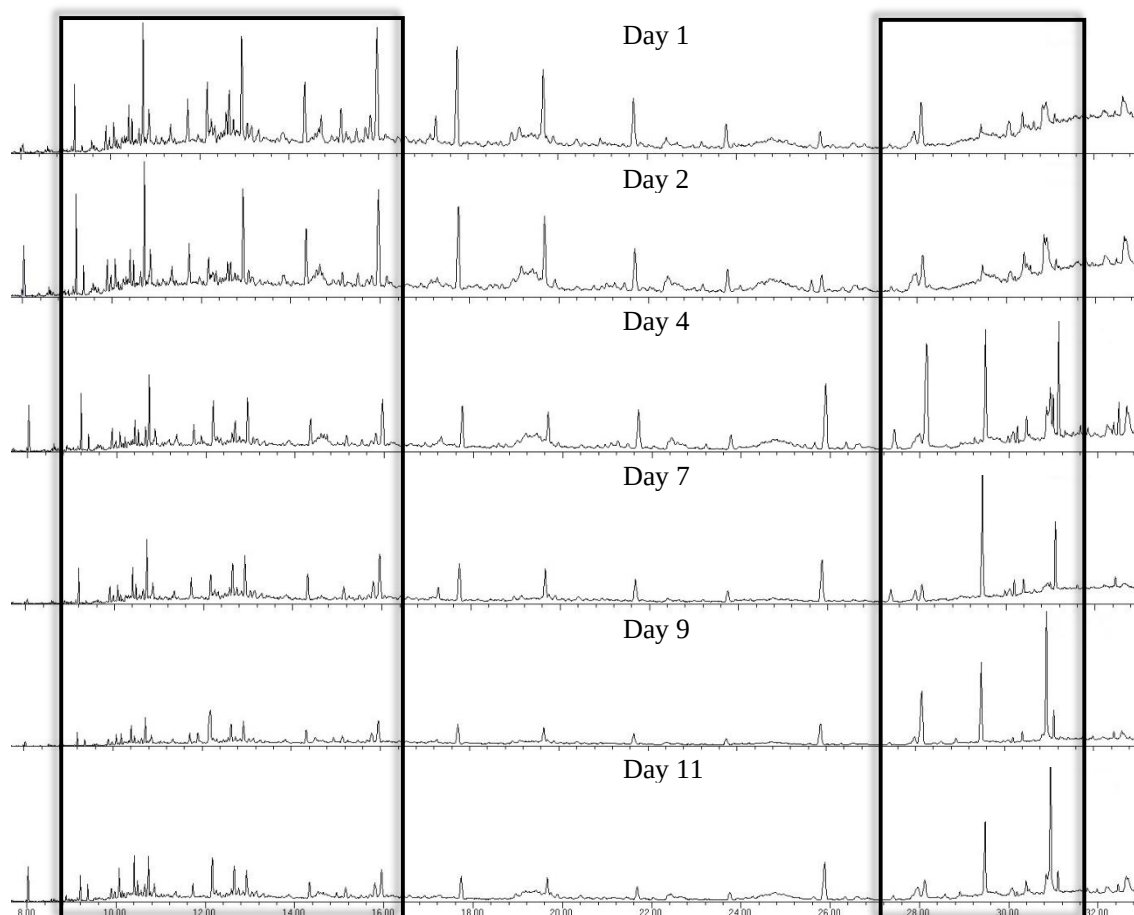


Figure 5.1: GC chromatograms of *C. vicina* larvae reared outdoors from Day 1 to Day 11

When the total percentage concentration of n-alkanes detected in the chromatograms was examined based on age (Appendix 12), the percentage concentrations of CHCs with shorter carbon chain lengths decreased from the immature stage to the post-feeding stage, and the percentage concentration of CHCs with longer carbon chain length increased. For example, the percentage concentrations of C24:H to C26:H decreased with age, while those of C27:H to C29:H increased. The percentage concentration of C29:H increased until Day 8, the last day of the third stage and decreased significantly after Day 9, the day the larvae entered the post-feeding stage and left the food source. Thus, the C29:H concentration was an important separation tool that separated the third instar and the post-feeding stages.

The change over age in the number of total n-alkanes and branched methyl alkanes in the larval development stage is given in Figure 5.2. Accordingly, the total number of n-alkanes increased until the 3rd instar larval stage. Although this number changes by Day 5, it remained almost stable throughout the 3rd instar and post-feeding stages. The number of branched methyl alkanes detected in the chemical profiles increased until the end of Day 3. A decrease was observed during the 3rd instar and post-feeding stages. On Days 10 and 11, there was a further increase in the number of branched methyl alkanes. The plot clearly shows that the total number of branched methyl alkanes almost doubled between the first instar larvae and post-feeding larvae. However, n-alkanes showed a stable distribution throughout the larval period.

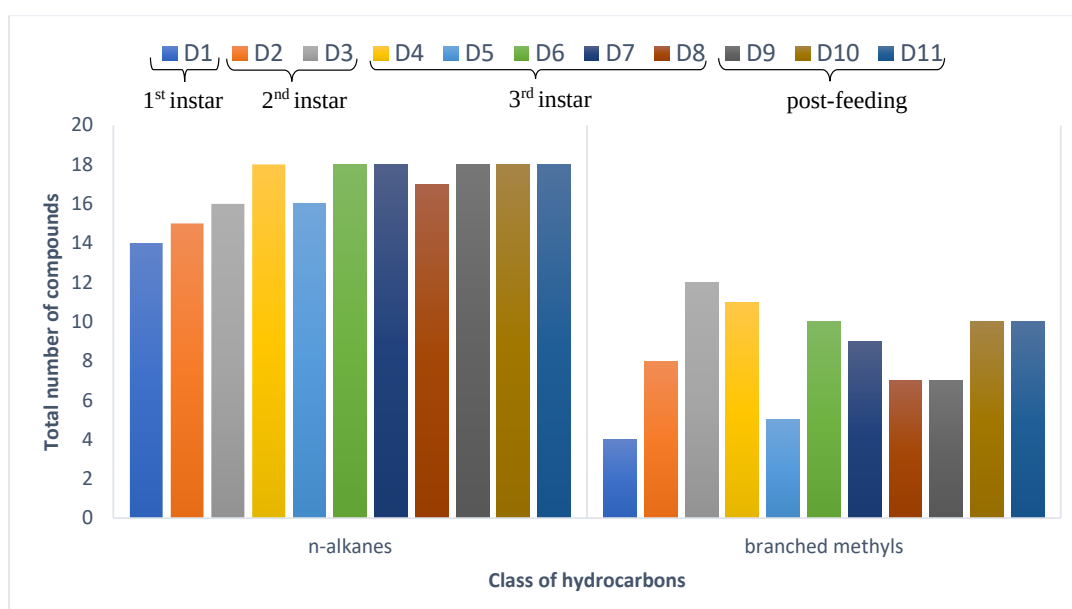


Figure 5.2: Plot showing the total number of n-alkanes and branched methyl alkanes identified over the larvae life cycle of *C. vicina* reared outdoors

In order to investigate the reason for the sudden decrease in the numbers of n-alkanes and branched methyl alkanes seen on Day 5, the outdoor weather temperatures that *C. vicina* larvae were exposed to were examined (Appendix 4). It was found that there was a 10°C decrease in daytime temperatures, which likely explains the sudden change in the CHC profiles of the 3rd stage larvae (i.e., the cuticle layer reaction to the temperature was reflected in the CHC profiles). Changes in the CHC profiles of larvae depending on age and temperature were evident in the chromatograms. The percentage concentration of

CHCs with higher boiling points and long carbon chains protects the larvae against water evaporation. According to the same temperature data, there was a changeover on Days 10 and 11 when the temperature decreased, whereby CHCs with long carbon chains were no longer needed; thus, their concentrations decreased.

The presence of age-related alterations on the chromatograms is inadequate to estimate the age of the larvae on its own, and compound-based age determination is a time-consuming and complicated procedure. As a consequence, a statistical analysis was performed on the GC-MS data that were acquired. Statistical analysis was initially carried out for all n-alkanes, alkenes and branched methyl alkanes detected in the reared *C. vicina* larvae. The PC2 and PC3 scores were utilised when generating the graph shown in Figure 5.3 since they offer the greatest differentiation between clusters. There was significant scatter, and although it was feasible to determine the age of the first five days, it was difficult to determine the age of the subsequent days with accuracy. The n-alkanes detected in the CHC profiles were highly stable, as shown in Figure 5.2, having the least variation. Therefore, a new PCA data set was prepared by eliminating the n-alkanes from the original data set.

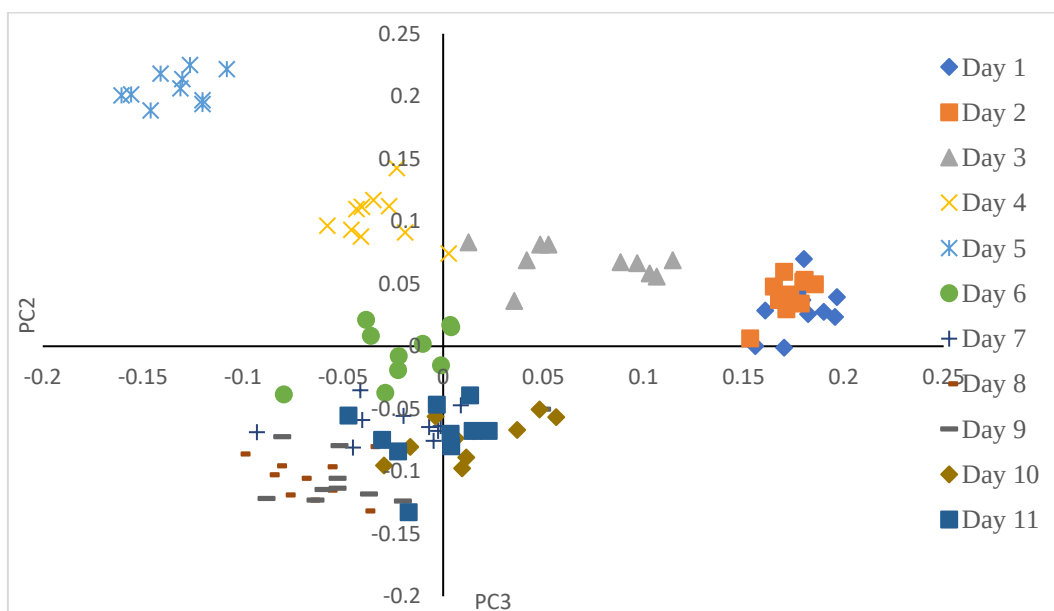


Figure 5.3: PCA plot showing PC2 vs PC3 for *C. vicina* larvae reared outdoors, using n-alkanes, alkenes and branched methyl alkanes

PCA was applied to a new dataset containing only alkenes and branched methyl alkanes to see if a more distinct separation could be visualised (Figure 5.4). PCA was performed using six principal components explaining 90.9% of the dataset variation, with the first four principal components being 63.0, 18.4, 5.4 and 4.1%, respectively. PC2 and PC3 scores were used to form the plot, as they were also used in the previous plot. Thus, the only variable was the elimination of n-alkanes and the effectiveness of eliminating n-alkanes was demonstrated.

Figure 5.4 indicates significantly improved clustering, which allowed ageing to be established with a much higher degree of accuracy. On the plot, the cluster days from Days 1 to 5 were clearly grouped by age in the lower area of the plot and can be aged to individual days. Separation was also obtained between Days 6, 7 and 8 (third instar) in the middle of the plot. Day 9 was highly scattered, and Days 10 and 11 (post-feeding) were clustered in the same group. On the PCA plot, the scatter seen on Day 9 shows age-related chemical changes in that age. There was a change in PC loading between Day 8 (third instar) and Day 10 (post-feeding instar). These scatters, appearing on Day 9, show the larvae between these two stages and show the differences between their chemical profiles.

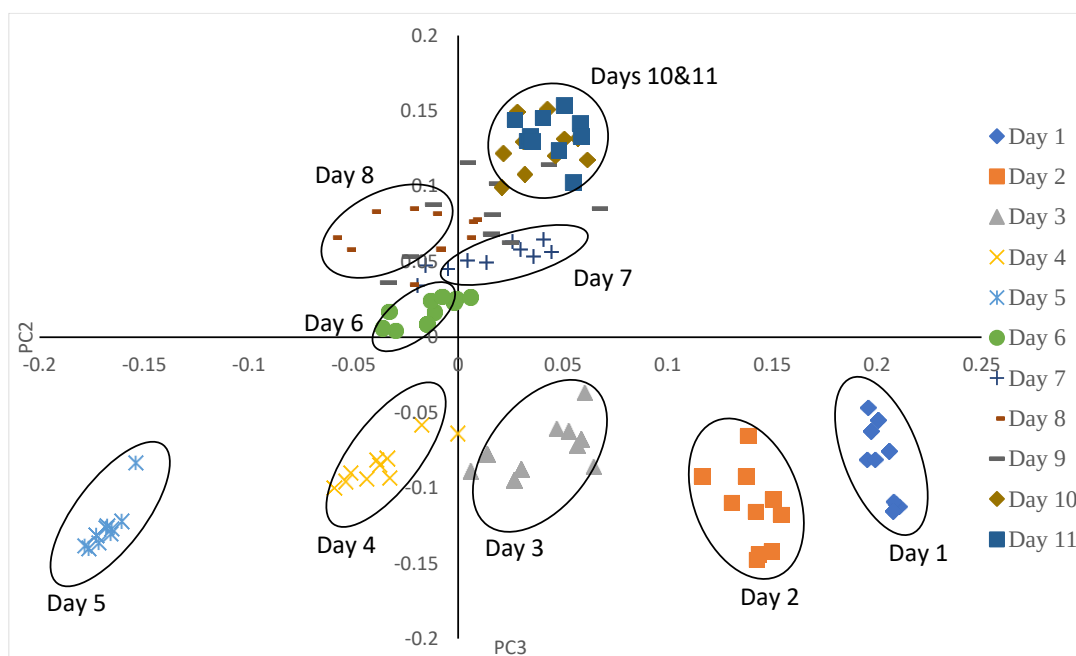


Figure 5.4: PCA plot showing PC3 against PC2 for *C. vicina* larvae reared outdoors, using alkenes and branched methyl alkanes only, with clustering days circled

As shown in Figure 5.4, although the PCA plot could be used to estimate a relative age between the clusters, the 3D PCA plot shown in Figure 5.5 was created by including a third score on top of the same dataset in order to obtain better visualisation between days, particularly during the post-feeding stage. PC5 score was added as the third dimension because it provides better separation with PC2 and PC3.

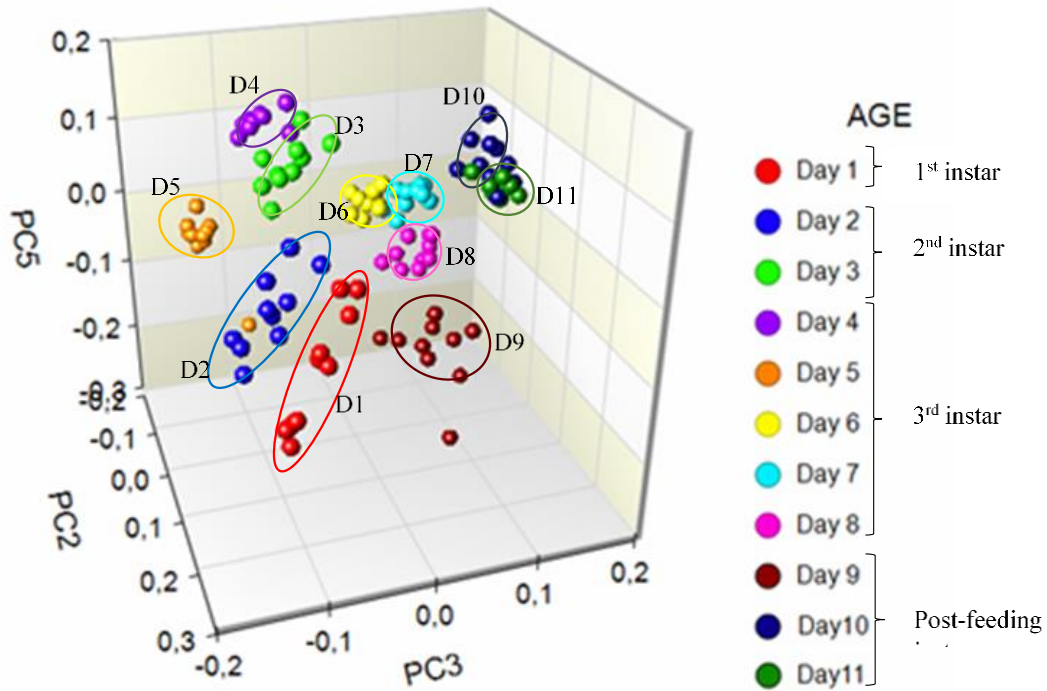


Figure 5.5: PCA 3D plot showing PC5 vs PC2 vs PC3 for *C. vicina* larvae reared outdoors, using alkenes and branched methyl alkanes only, with clustering days circled

Age estimation for *C. vicina* larvae reared outdoors was achieved based on changes in CHC profiles that occur with age. In entomological methods, the larvae are separated into their development stages according to the spiracular slits of the posterior and aged with ADD/H. However, since the data used in previous studies were not obtained under fluctuating temperatures in the field, their accuracy was controversial. Thanks to the CHC analysis and 3D PCA plot, the developmental stages of outdoor reared *C. vicina* larvae were clustered and aged on a day-by-day basis. In the studies conducted so far, this degree of accuracy has not been achieved for the larval stages at which it can be aged to the day. These results provide a very promising springboard for future studies.

5.2.1.1.2 Control group at constant temperature ($25\pm1^{\circ}\text{C}$)

This study essentially extends the experimental work of Moore [40], who conducted her studies at a constant temperature in controlled laboratory conditions. In order to take the study one step further, this thesis examined the effects of environmental factors and fluctuated temperature on the CHC profiles by modelling their chemical profiles when reared outdoors. For this reason, *C. vicina* larvae reared in a $25\pm1^{\circ}\text{C}$ incubator were set up as a control group and were used to compare with the chemical profiles of the sample sets reared outdoors. For detailed information on experimental procedures, Moore's [40] studies can be consulted.

In the chromatograms of control group *C. vicina* larvae, a total of 24 CHC compounds were identified. These CHCs detected by GC and identified by MS were applied to PCA for age estimation. The total percentages of CHCs of all compounds (Table 5.2) were n-alkanes (71%) and branched methyl alkanes (29%) in Days 1 to 9 of all larval developmental stages. A small number of alkenes were detected, but their percentage peak areas were less than 0.5%. Thus, they were not included in the comparisons. The chain lengths of CHCs ranged from C16:H to C33:H. The predominant group in concentration and number was n-alkanes in the chemical profiles.

Table 5.2: CHC compounds extracted from *C. vicina* larvae reared indoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1	C16	5.89±3.93	1.24±0.88	tr	1.24±1.07	1.69±1.36	1.13±0.44	tr	tr	tr
2	C17	tr	0.93±0.59	tr	0.90±0.74	1.26±0.58	0.98±0.37	tr	0.84±0.30	tr
3	C18	8.09±6.82	3.93±2.43	3.63±2.91	2.84±2.44	4.29±2.02	3.41±1.18	2.85±1.23	2.62±1.11	2.15±0.62
4	7-MeC18	tr	1.72±0.92	2.20±1.67	1.70±1.64	2.49±1.12	1.94±0.57	1.93±0.60	2.24±0.77	1.87±0.70
5	C19	tr	2.46±1.33	2.80±2.14	1.69±1.53	2.74±1.08	2.22±0.63	2.27±0.78	2.19±0.72	2.09±0.56
6	3-MeC19	tr	tr	tr	tr	tr	1.42±0.56	tr	2.02±1.07	tr
7	C20	tr	5.27±2.14	5.58±2.30	2.84±1.76	4.57±1.89	4.22±1.04	5.22±1.58	3.67±1.23	3.87±0.51
8	C21	9.52±7.54	5.35±2.34	4.73±1.63	2.69±1.64	4.07±1.73	4.16±0.89	4.54±1.09	3.14±0.93	3.24±0.61
9	4-MeC21	tr	tr	tr	tr	tr	1.30±0.35	tr	tr	tr
10	3-MeC21	tr	1.19±0.35	tr	tr	tr	4.76±1.66	14.48±11.64	16.00±3.48	tr
11	C22	6.41±11.88	9.00±2.91	9.36±3.31	3.96±2.62	7.08±2.59	7.76±1.77	8.73±1.99	5.38±1.35	6.37±0.87
12	C23	4.27±3.81	10.06±3.44	9.86±3.65	4.60±2.44	7.20±2.73	8.57±1.95	tr	5.85±1.32	6.79±1.05
13	C24	2.59±1.25	9.35±3.15	9.21±3.59	4.43±2.35	7.17±3.93	8.29±1.83	tr	5.65±1.82	6.23±1.82
14	3-MeC24	5.21±3.04	1.44±0.46	tr	tr	tr	tr	8.99±3.05	tr	tr
15	C25	12.83±5.86	10.75±7.02	7.45±2.90	4.22±2.12	5.20±1.88	5.48±1.42	5.65±1.29	3.66±0.81	4.59±0.61
16	C26	7.61±7.26	3.84±1.81	3.41±1.55	1.89±0.94	2.75±1.10	3.20±0.72	18.14±13.24	18.99±3.81	2.29±0.45
17	C27	12.78±5.46	7.15±3.00	9.35±4.00	9.89±4.93	4.93±2.02	6.56±1.82	6.47±2.08	4.91±1.49	7.13±1.68
18	3-MeC27	3.37±1.81	4.35±2.13	2.29±1.17	2.11±1.19	1.60±1.03	1.06±0.33	tr	tr	tr
19	C28	8.25±10.47	8.39±4.96	8.47±5.30	4.96±2.96	7.24±5.32	7.95±1.94	5.49±2.63	4.64±1.43	7.94±1.61
20	C29	13.16±5.01	9.25±5.55	10.81±5.12	24.75±13.68	17.81±6.79	14.96±7.44	15.24±7.25	13.10±3.31	31.99±6.23
21	3-MeC29	tr	1.60±1.16	1.29±1.11	1.84±1.12	1.29±0.59	0.94±0.37	tr	tr	0.86±0.19
22	C30	tr	tr	tr	4.32±2.53	5.18±3.39	5.51±1.70	tr	2.36±1.19	3.07±1.47
23	C31	tr	2.74±1.02	7.50±3.11	15.88±8.90	8.75±3.60	3.23±0.99	tr	2.72±1.07	9.51±2.56
24	C33	tr	tr	2.04±0.81	3.24±1.84	2.67±2.05	0.93±0.37	tr	tr	tr

tr = Trace amount detected (<0.5%)

Figure 5.6 shows the GC chromatograms of the immature ages (Days 1, 2 and 3) of *C. vicina* larvae reared under controlled constant $25\pm1^{\circ}\text{C}$ temperature and reared under fluctuating temperatures in the field. The marked region shows the variation in concentrations of CHCs with 16 to 21 carbon chains. In the chromatograms, it was noticed that the percentage concentration of these CHCs was lower in larvae reared under constant temperature. In contrast, they were relatively higher in larvae reared outdoors under ambient environmental conditions. Greater concentrations of these compounds were found in the larvae exposed to fluctuating temperature, indicating the effect of variable temperature on CHC profiles.

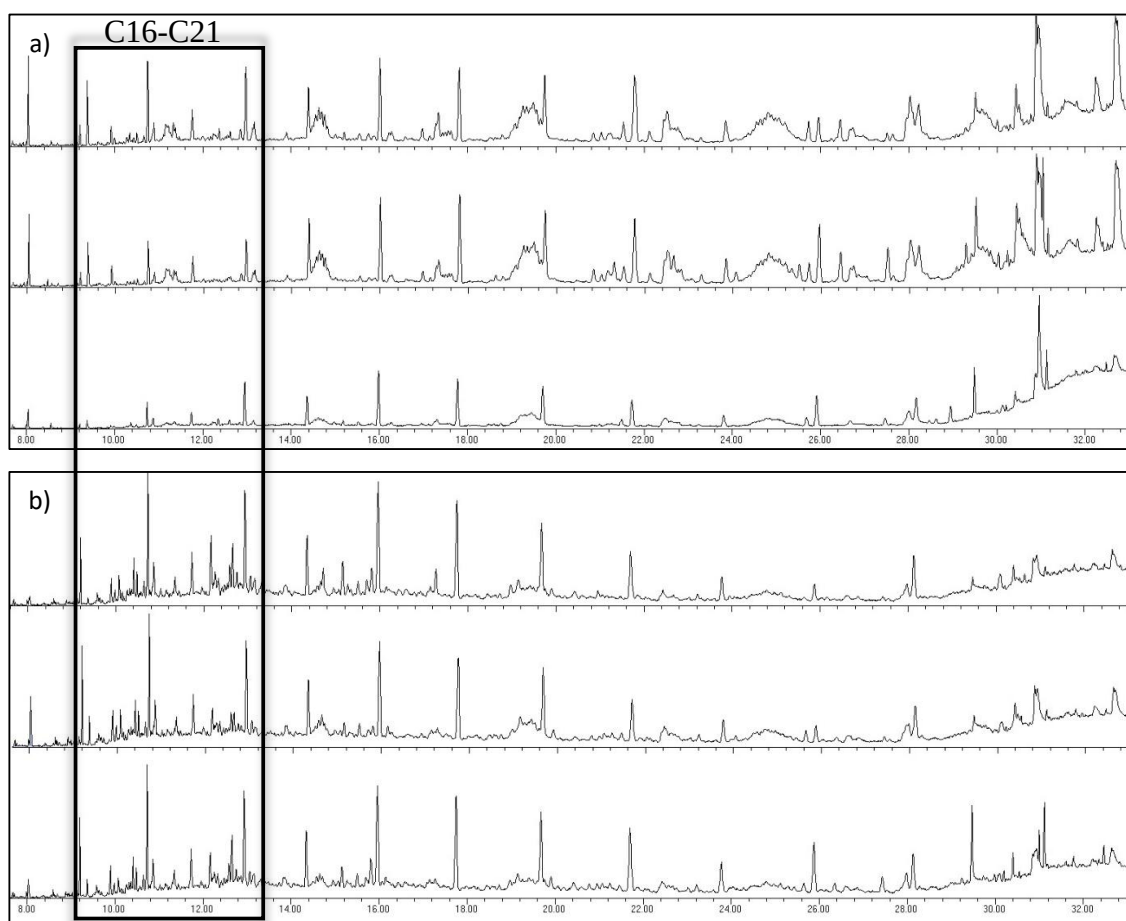


Figure 5.6: GC chromatograms of *C. vicina* larvae reared in the UK for Days 1, 2 and 3, respectively, reared a) indoors, b) outdoors

The total percentage concentrations of n-alkanes and branched methyl alkanes detected during the whole larval development stage for *C. vicina* were higher in larvae reared outdoors. When the change in the total percentage concentrations of n-alkanes and branched methyl alkanes in the CHC profiles on the first and last day of the larval development stage of *C. vicina* larvae reared indoors and outdoors were examined (Appendix 13), it was found that the total concentration of n-alkanes increased by 6% between the first and last larval development days and the total concentration of branched methyl alkanes decreased by 6% in larvae reared indoors. In contrast, in outdoor-reared larvae, while the total concentration of n-alkanes decreased by 12% between the first and last larval days, the total concentration of branched methyl alkanes increased by 7%.

Apart from investigating the effects of fluctuating and constant temperature on CHC profiles, age estimates of larvae reared at constant temperature were also carried out as a preliminary study as part of this study. Figure 5.7 shows the 3D PCA plot of *C. vicina* larvae extractions. The PCA data was created for Days 1 to 9. Following the procedure described in the preceding section, n-alkanes were removed, and branched methyl alkanes with more variation were included in the PCA calculation in order to achieve a sufficient differentiation between larval ages. PCA was performed using six main components explaining 90.9% of the data set variation, with the first three principal components being 74.1, 9.3 and 7.5%, respectively. PC1, PC2 and PC3 scores were used to form the plot because they provide the clearest distinction.

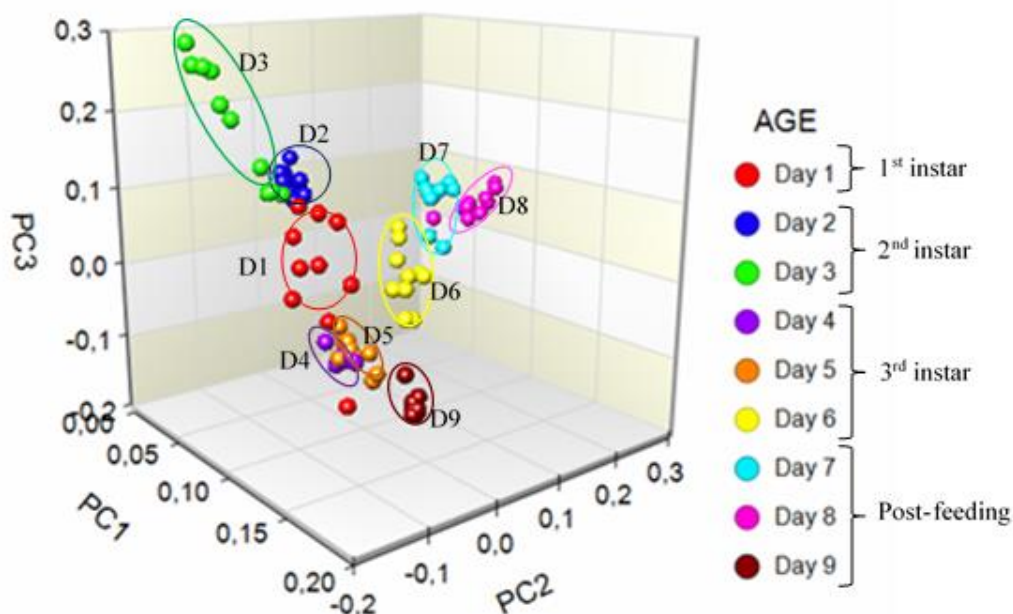


Figure 5.7: PCA 3D plot showing PC3 vs PC1 vs PC2 for *C. vicina* larvae reared indoors, using branched methyl alkanes only, with clustering days circled

The PCA plot in Figure 5.7 provided the day age estimation for the entire larval developmental period. There was a clear separation between individual days. Clusters representing Day 1 (first instar) and Days 2 and 3 (second instar) were clearly separated from other stages. Days 4, 5 and 6 (third instar) and Days 7, 8 and 9 (the post-feeding larvae) had relatively similar chemical profiles and morphological characters within their developmental stages. The clusters, on the other hand, did not scatter, and the age in days of larvae at the same developmental stage could be separated from others. The combination of CHC analysis and 3D PCA plots allows an age estimation for *C. vicina* larvae reared indoors based on the age-related changes in CHC profiles.

5.2.1.2 *L. sericata* larvae

5.2.1.2.1 Fieldwork study

In GC chromatograms, a total of 33 CHC compounds were detected in all larval developmental stages of *L. sericata* larvae reared outdoors. The identified CHC compounds are given in Table 5.3. The predominant group in CHC profiles are n-alkanes in terms of total concentration and number. The total CHC concentrations of CHC profiles

were n-alkanes (55%), alkenes (%6) and branched methyl alkanes (39%). Chain lengths ranged from C16:H to C33:H.

The number of n-alkanes increased from Day 1 to Day 3 and remained constant between Day 4 and Day 6. There was a slight increase in the total number of n-alkanes between Days 7 and 9 (the post-feeding stage). It was found that the highest number of n-alkanes were found on Day 9. While the number of branched methyl alkanes was deficient in first instar larvae, this number increased in second instar larvae and then continued to decrease until Day 6. The number of branched alkanes increased again in the post-feeding stage. The 3-MeC27 compound was detected in the CHC profiles of Days 2, 3 and 4. The 7-MeC25 compound was detected in the profiles of Days 2 and 3. The 7-MeC29 compound was detected only in the Day 2 profile, while the 3-MeC29 compound was detected only in the Day 3 profile. These branched methyl alkanes can be considered as the age indicator for the mentioned days.

Table 5.3: CHC compounds extracted from *L. sericata* larvae reared outdoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
1	C16	2.21±0.47	1.77±0.44	2.29±0.33	2.63±0.58	2.23±1.84	0.95±0.49	0.66±0.59	tr	tr
2	C17	1.22±0.34	1.25±0.45	1.60±0.28	1.67±0.47	1.62±1.03	tr	0.62±0.38	tr	tr
3	7-MeC17	0.78±0.33	0.88±0.34	tr	tr	1.06±0.51	tr	tr	tr	tr
4	3-MeC17	1.79±0.34	1.43±0.64	2.08±0.52	1.64±0.64	2.65±1.42	1.67±1.21	1.54±0.48	0.87±0.29	0.51±0.17
5	C18	5.53±1.09	5.18±1.51	5.98±1.19	6.22±1.88	6.34±3.46	3.73±2.39	2.71±1.12	1.73±0.47	1.28±0.37
6	7-MeC18	2.65±0.64	2.62±0.98	3.54±1.04	2.67±1.05	3.60±1.83	2.83±2.02	1.93±0.70	1.18±0.38	0.90±0.33
7	C19	3.16±0.81	2.61±0.90	3.38±0.83	3.08±1.01	3.53±1.98	2.64±1.85	1.68±0.58	1.16±0.42	0.82±0.30
8	9+11-MeC19	2.27±1.32	2.14±1.32	1.80±0.60	2.46±0.54	tr	tr	2.86±1.46	0.82±0.48	3.14±2.21
9	3-MeC19	1.54±0.57	1.38±0.44	1.77±0.50	1.63±0.55	1.88±0.99	1.48±1.06	0.74±0.13	0.65±0.16	0.51±0.17
10	Eicosene*	3.85±1.07	3.15±1.48	2.82±1.06	3.11±1.27	2.64±1.27	2.23±1.38	2.31±0.44	1.53±0.27	1.03±0.26
11	C20	7.17±1.38	5.61±1.45	6.24±1.40	6.71±1.89	7.35±3.34	5.60±3.39	3.14±0.73	2.34±0.41	1.60±0.40
12	3-MeC20	tr	tr	tr	tr	tr	tr	tr	0.77±0.45	0.52±0.16
13	C21	5.10±1.29	3.49±0.93	3.94±0.77	4.38±0.97	4.77±2.33	4.17±2.03	2.23±0.47	1.56±0.20	1.10±0.23
14	5-MeC21	tr	tr	tr	tr	tr	tr	0.97±0.23	0.74±0.11	0.53±0.14
15	3-MeC21	tr	tr	tr	1.66±0.33	4.34±2.33	tr	1.54±0.74	1.02±0.52	0.51±0.14
16	Docosene*	tr	tr	tr	tr	2.66±1.50	tr	1.65±0.43	0.98±0.15	0.69±0.17
17	C22	9.94±2.04	7.29±1.87	7.49±1.54	8.90±2.29	9.83±4.02	8.34±4.54	4.76±1.08	3.20±0.46	2.14±0.33
18	C23	10.28±1.91	7.49±1.95	7.20±1.29	8.39±1.76	8.96±3.54	8.28±3.86	4.49±0.87	3.17±0.46	2.04±0.23
19	C24	9.61±1.93	6.72±2.05	6.55±1.82	8.08±1.88	8.65±4.01	8.71±4.19	4.07±1.14	2.53±0.72	1.73±0.22
20	C25	6.69±1.16	7.18±2.61	5.51±1.10	6.30±1.40	7.22±2.85	7.00±3.16	3.44±1.00	2.52±0.61	2.19±0.61
21	7-MeC25	tr	2.42±1.27	1.66±0.59	tr	tr	tr	tr	tr	tr
22	C26	3.08±0.74	2.40±0.71	2.21±0.43	2.50±0.58	2.63±0.87	2.66±1.34	1.45±0.29	2.52±1.01	0.84±0.15

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8	DAY 9
23	C27	2.63±0.51	5.71±2.05	4.05±1.12	3.77±1.23	3.29±1.08	5.37±2.52	3.93±0.90	6.26±1.67	9.76±2.69
24	3-MeC27	tr	1.90±0.71	1.49±0.62	1.36±0.42	tr	tr	tr	tr	tr
25	C28	7.08±2.43	4.69±1.78	3.85±1.01	5.35±0.94	3.91±2.55	6.87±2.68	4.29±1.49	3.30±0.87	3.24±0.60
26	C29	13.42±2.56	11.55±3.73	9.21±2.63	9.25±2.95	5.62±3.97	14.39±4.49	22.75±4.94	31.80±4.60	39.45±4.35
27	7-MeC29	tr	2.81±1.25	tr	tr	tr	tr	tr	tr	tr
28	3-MeC29	tr	tr	1.92±0.75	tr	tr	tr	tr	tr	tr
29	C30	tr	3.70±1.16	3.19±0.68	3.91±1.25	tr	tr	4.27±1.07	3.76±0.27	3.41±0.40
30	C31	tr	4.63±1.96	7.55±2.92	4.31±1.70	4.15±2.05	10.18±5.21	18.85±5.22	20.28±2.15	18.43±1.78
31	11+13-MeC31	tr	tr	tr	tr	tr	tr	tr	tr	tr
32	C32	tr	tr	tr	tr	tr	tr	tr	1.70±0.40	0.97±0.27
33	C33	tr	tr	2.67±0.98	tr	1.06±0.51	2.88±1.39	3.13±0.97	2.91±0.32	2.30±0.42

tr = Trace amount detected (<0.5%)

* Double bond positions were not determined

Figure 5.8 shows GC chromatograms of *L. sericata* larvae reared outdoors from Days 1, 2, 4, 7 and 9. The areas marked on the chromatograms highlighted the significant chemical changes visible in the chemical profiles. Age-related changes were evident in the chromatograms of *L. sericata* larvae. As the larvae developed, their chemical profiles changed, and the percentage concentration of short carbon chain CHCs was replaced with long carbon chain CHCs. The percentage concentrations of C16:H to C25:H n-alkanes increased, while a considerable decrease was observed after Day 6. In contrast, the percentage concentrations of C27:H to C33:H n-alkanes increased significantly. Although the age of the larvae could not be explicitly predicted by examining the chromatograms, it was possible to estimate the development stage by observing their chromatogram.

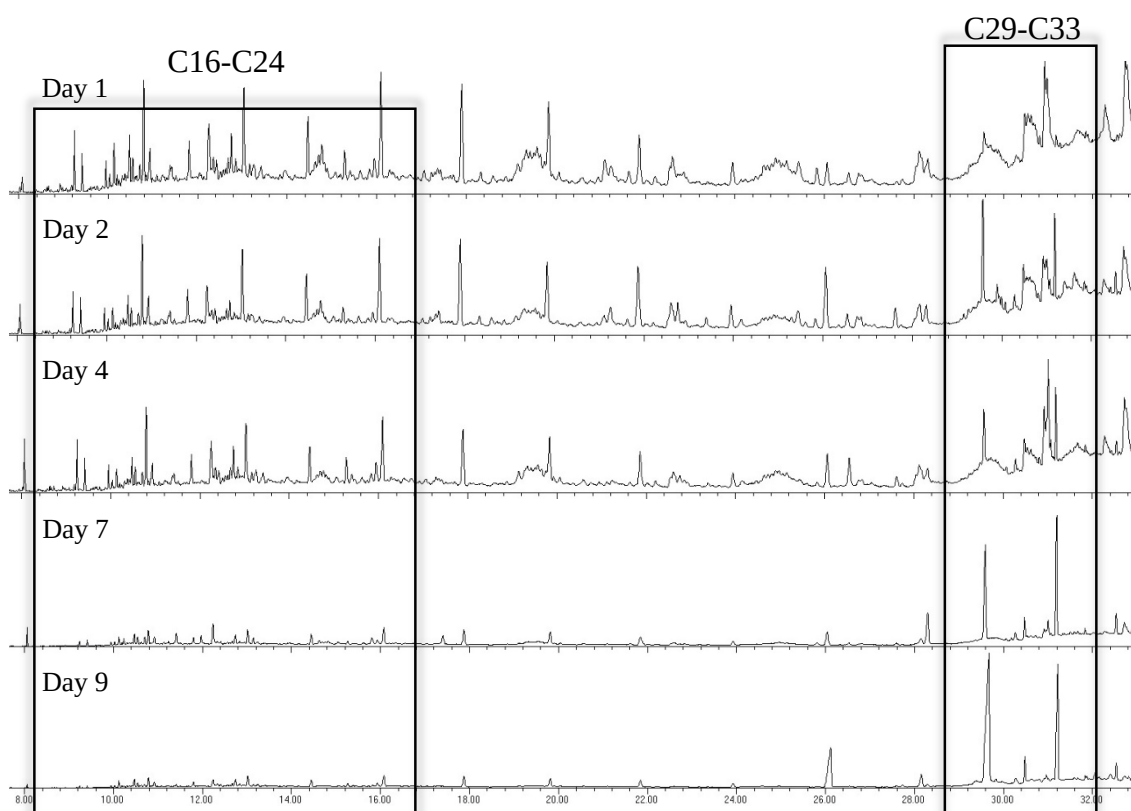


Figure 5.8: GC chromatograms of *L. sericata* larvae reared outdoors from Days 1, 2, 4, 7 and 9

Although the number of CHCs varies, their concentration in the chemical profiles is also an important variable and can sometimes increase or decrease independently of the number. This situation was investigated, and the results shown in Figure 5.9 indicate that the total concentration percentages of n-alkanes and branched methyl alkanes by age during the larval period are given. Total n-alkane concentration on Day 1 was higher; however, when the larvae developed and entered the second instar stage (Days 2 and 3), the total concentration of n-alkanes decreased. After Day 2, the percentage concentration of n-alkanes changed in proportion to the development of the larvae. Although the number of branched methyl alkanes increased, their percentage concentration decreased from the second instar larvae to the post-feeding larvae. It is possible to conclude that the proportion of total n-alkanes remained almost constant, albeit with minor variations, throughout time.

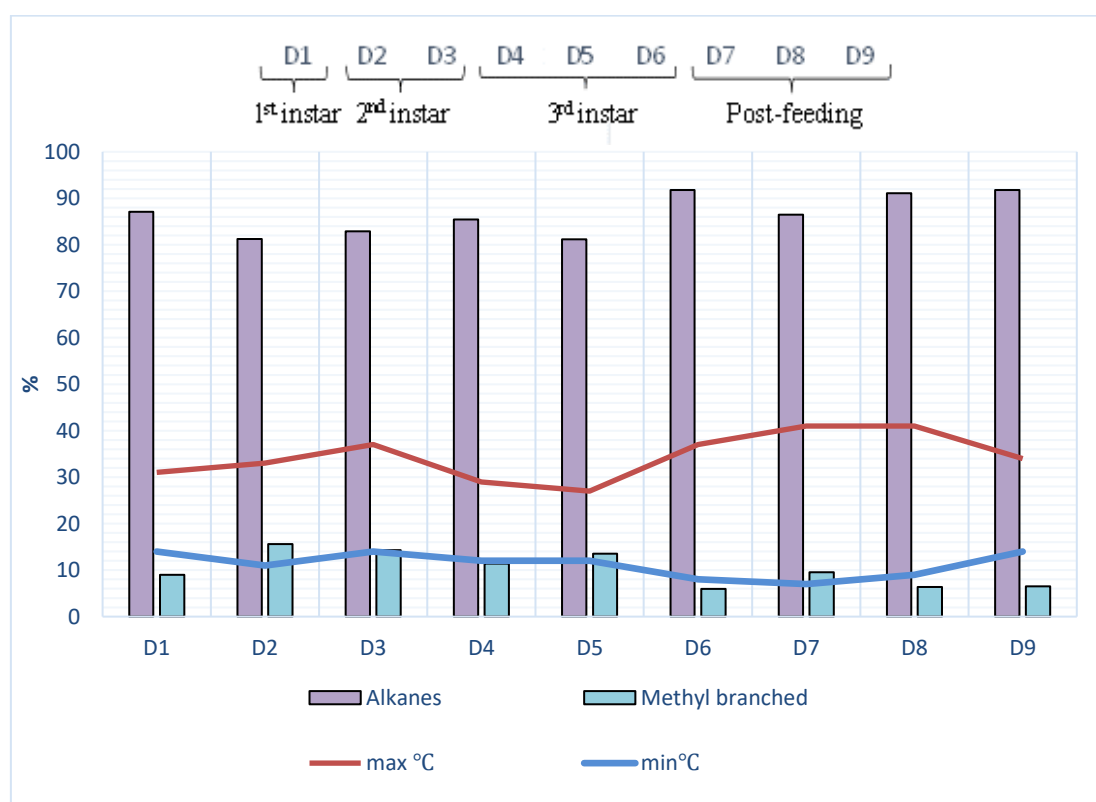


Figure 5.9: Plot showing the total percentage concentration of n-alkanes and branched methyl alkanes present, and max and min temperature values over the larvae life cycle of *L. sericata* reared outdoors

As seen in Figure 5.9, the n-alkane concentration of Day 6 larvae increased by 10% compared to the previous days. When the temperature data to which *L. sericata* larvae were exposed were examined, while the temperature difference between day and night was less on Days 4 and 5, this difference increased on Day 6 and continued to increase on other days. An increase in n-alkane concentration and a decrease in branched methyl alkane with increasing temperature was an expected reaction due to the boiling points.

The PCA plot was formed using the CHC compounds (Table 5.3) to obtain a good separation between clusters. However, due to intercluster scattering, as observed in *C. vicina* larvae, a plot with which a precise age estimation could not be obtained. Therefore, n-alkanes were eliminated as they were more stable, and the second set of PCA was created using alkenes and branched methyl alkanes that changed more with age. The PCA plot in which only alkenes and branched methyl alkanes were included is given in Figure 5.10, formed for *L. sericata* larvae reared outdoors from Day 1 to Day 9. PC2, PC3 and PC6 were used for the 3D axis of the plot. As with the previous PCA plots, 3D was preferred as it provides better separation. It was performed using six main components explaining 82.2% of the data set variation, with the first three principal components being 46.5, 26.6 and 9.1%, respectively.

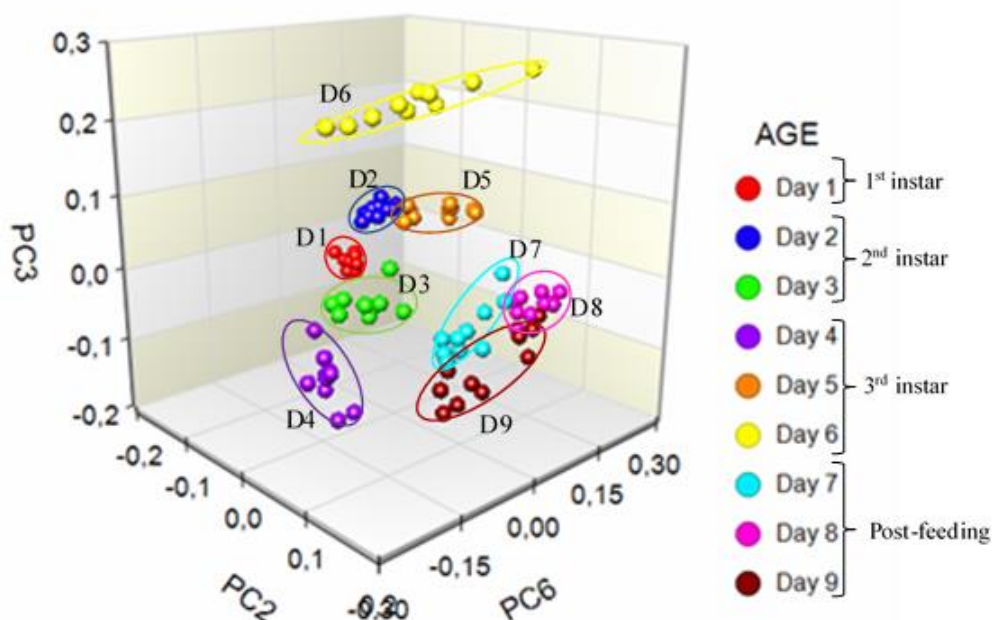


Figure 5.10: PCA 3D plot showing PC3 vs PC2 vs PC6 for *L. sericata* larvae reared outdoors, using alkenes and branched methyl alkanes only, with clustering days circled

Age clusters from Day 1 to Day 6 were clearly separated from each other on the PCA plot, especially for Days 7, 8 and 9, representing the post-feeding stage larvae, which have similar physiological and chemical profiles. On the PCA plot, the accuracy is higher as there is no scattering in the clusters and age estimates can be made even though they are closely located as age clusters.

It was discovered that the CHC analysis technique and the 3D PCA plot, which offers a day-to-day age estimate for *L. sericata* larvae reared outdoors by exposure to fluctuating temperatures, produced significant findings in this study. The results would be beneficial to forensic investigators in making their PMI_{min} conclusions in the future.

5.2.1.2.2 Control group at constant temperature (25±1°C)

In total, *L. sericata* yielded a profile of 34 CHCs peaks from Days 1 to 8 (Table 5.4). The total CHC concentrations of CHC profiles were n-alkanes (50%) and branched methyl alkanes (50%) during the larval period. The chain lengths ranged from C17:H to C33:H. The alkenes were detected, but their concentrations were less than 0.5%. Thus, alkenes were not included in the comparisons. Although equal numbers of n-alkanes and methyl alkanes were detected in CHC profiles, the predominant group was n-alkanes in concentration.

Table 5.4: CHC compounds extracted from *L. sericata* larvae reared indoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8
1	C17	tr	tr	0.68±0.33	1.02±0.95	tr	tr	tr	tr
2	7-MeC17	tr	tr	0.63±0.19	tr	tr	tr	tr	tr
3	3-MeC17	tr	tr	0.95±0.28	tr	tr	tr	0.69±0.16	1.02±0.41
4	C18	2.69±1.33	1.39±0.91	3.00±1.20	4.18±3.46	2.18±1.34	2.19±1.29	2.17±0.72	2.99±1.13
5	7-MeC18	1.67±0.72	0.83±0.34	2.09±0.73	1.90±1.02	1.59±0.46	1.24±0.63	1.30±0.26	2.72±1.16
6	3-MeC18	tr	tr	1.89±1.66	tr	tr	tr	tr	tr
7	C19	1.99±0.72	1.02±0.47	2.48±0.81	2.28±1.02	1.77±0.64	1.42±0.55	1.66±0.31	2.72±1.15
8	3-MeC19	tr	tr	2.49±1.15	tr	tr	tr	tr	tr
9	C20	5.93±1.72	3.14±1.00	5.42±1.31	4.77±1.39	4.37±1.00	2.75±0.69	3.37±1.17	3.61±0.78
10	C21	4.93±1.62	2.54±0.66	4.93±1.12	3.87±1.04	3.99±0.94	2.38±0.45	2.93±0.69	3.05±0.48
11	4-MeC21	tr	tr	2.19±0.96	tr	tr	tr	tr	tr
12	C22	9.82±2.62	5.18±1.34	8.47±3.11	7.66±1.60	8.68±2.76	4.60±0.84	6.05±1.50	6.41±0.95
13	3-MeC22	1.39±0.59	0.73±0.48	tr	tr	tr	tr	tr	tr
14	C23	11.99±3.73	7.19±2.61	9.28±2.09	7.62±1.71	8.19±1.51	4.59±0.83	5.85±1.40	5.79±1.07
15	11+13-MeC23	2.26±1.08	1.28±1.06	tr	tr	tr	tr	tr	tr
16	C24	10.81±4.24	5.36±1.98	9.96±2.94	8.65±3.44	9.93±1.94	5.22±1.91	6.10±1.85	5.72±0.74
17	7-MeC24	tr	tr	2.07±0.79	1.76±0.64	2.09±0.76	1.30±0.60	tr	tr
18	3-MeC24	1.35±0.69	tr	tr	tr	tr	tr	tr	tr
19	C25	10.65±4.48	10.02±4.01	6.80±1.26	7.16±2.54	6.54±1.56	4.96±2.45	4.05±0.96	3.43±0.41
20	11+13-MeC25	6.67±4.18	4.58±3.68	5.11±2.48	tr	tr	tr	tr	tr
21	7-MeC25	tr	2.34±0.84	3.28±1.82	tr	tr	tr	tr	tr
22	3-MeC25	tr	1.34±0.50	1.43±0.30	tr	tr	tr	tr	tr

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 6	DAY 7	DAY 8
23	C26	3.19±0.94	2.56±0.91	2.89±0.69	2.66±0.67	2.67±0.49	1.59±0.32	2.04±0.42	13.33±7.15
24	3-MeC26	tr	tr	2.06±0.85	2.25±0.94	2.51±0.70	1.38±0.58	1.36±0.29	1.26±0.19
25	C27	6.36±3.86	15.18±17.16	2.49±0.62	5.81±2.07	4.54±1.41	5.05±1.37	4.48±1.02	3.00±0.54
26	3-MeC27	tr	3.27±2.17	1.42±0.85	1.88±0.84	tr	1.00±0.20	tr	tr
27	C28	7.32±4.17	4.78±4.00	7.21±3.05	7.04±3.03	8.15±2.55	4.46±1.52	4.79±1.53	3.89±0.58
28	C29	10.58±4.56	20.23±24.56	4.39±2.24	9.04±4.06	9.80±2.68	16.00±3.72	22.86±6.53	20.05±2.86
29	3-MeC29	tr	1.75±1.20	tr	2.11±0.89	2.13±0.93	1.64±0.91	tr	tr
30	C30	tr	2.55±2.07	4.65±2.86	5.35±2.55	5.15±0.95	4.73±1.90	4.26±1.17	4.27±1.39
31	C31	tr	2.74±2.13	1.73±0.27	7.14±4.69	8.87±2.48	21.30±6.48	17.20±4.34	12.39±2.70
32	3-MeC31	tr	tr	tr	tr	tr	2.28±0.90	3.01±1.48	tr
33	C32	tr	tr	tr	3.96±2.03	4.81±1.45	4.27±1.87	2.81±1.03	2.41±1.06
34	C33	tr	tr	tr	1.89±0.99	2.03±0.45	5.66±1.44	3.02±0.92	1.94±0.46

tr = Trace amount detected (<0.5%)

Figure 5.11 shows GC chromatograms of *L. sericata* larvae reared outdoors under fluctuating temperatures and indoors at a controlled constant ($25\pm1^{\circ}\text{C}$) temperature for Days 1 and 2 and the last larval day just before the pupae period. It can be seen that the larvae exposed to fluctuating outdoor temperatures had higher concentrations of both short-chain CHCs than the larvae exposed to a constant indoor temperature. In the chromatogram of the larvae reared at a constant temperature, the percentage concentration of n-alkanes was higher than branched alkanes. For both the larvae reared outdoors and indoors on the last day of the larval development, the CHCs with short-chain length were replaced with CHCs with longer chain length just before the pupae stage.

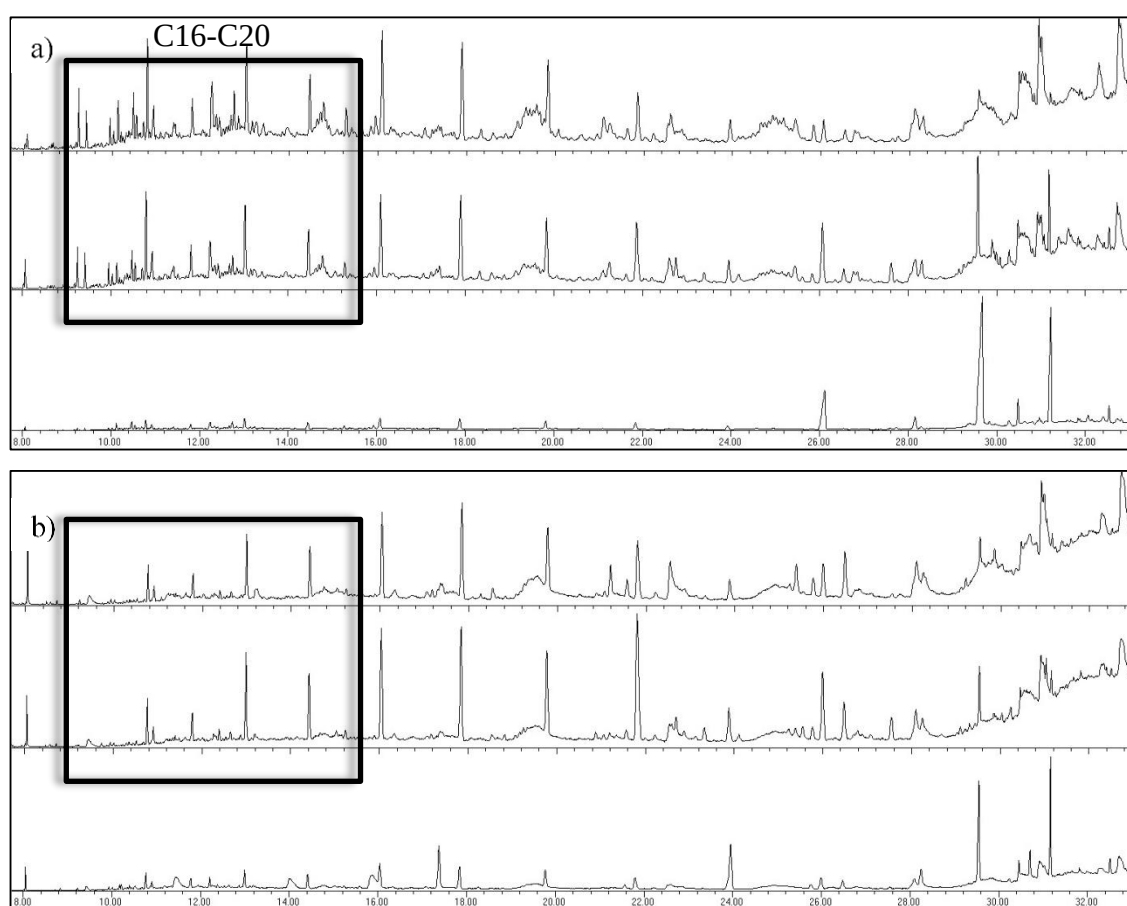


Figure 5.11: GC chromatograms of *L. sericata* larvae for Days 1, 2 and the last day of the post-feeding stage, respectively, reared a) outdoors b) indoors

The detected percentage concentration of n-alkanes in larvae reared under fluctuating temperatures was more significant than in larvae reared at constant temperatures. On the other hand, the percentage concentration of branched alkanes in larvae reared indoors was similar to that of those reared outdoors. The distribution of total alkane and branched methyl alkane peak areas according to indoor and outdoor rearing shows the effect of temperature. It can be concluded that the larvae reared outdoors have a higher concentration of n-alkanes to protect the organism against fluctuating temperature.

When comparing the total percentage concentration of n-alkanes and branched methyl alkanes of *L. sericata* larvae reared outdoors and indoors for Day 1 and the last larval day (Appendix 14), there was a 5% increase in the total n-alkane concentration and a 2% decrease in the total branched methyl alkane concentration between the first and last larval days of the larvae reared outdoors. On the other hand, for larvae reared at the constant temperature, there was an 8% increase in the total n-alkane concentration and an 8% decrease in the total methyl alkane concentration between those larval days.

Age estimates of *L. sericata* larvae reared at constant temperature were also carried out as a preliminary study. The 3D PCA plot (Figure 5.12) was generated by including the n-alkanes and branched methyl alkanes identified from chromatograms of *L. sericata* larvae reared at $25\pm 1^{\circ}\text{C}$. PC2, PC3 and PC5 scores were used to generate the three axes of the plot. PCA was performed using six main components explaining 92.8% of the data set variation, with the first three principal components being 75.8, 14.1 and 2.9%, respectively.

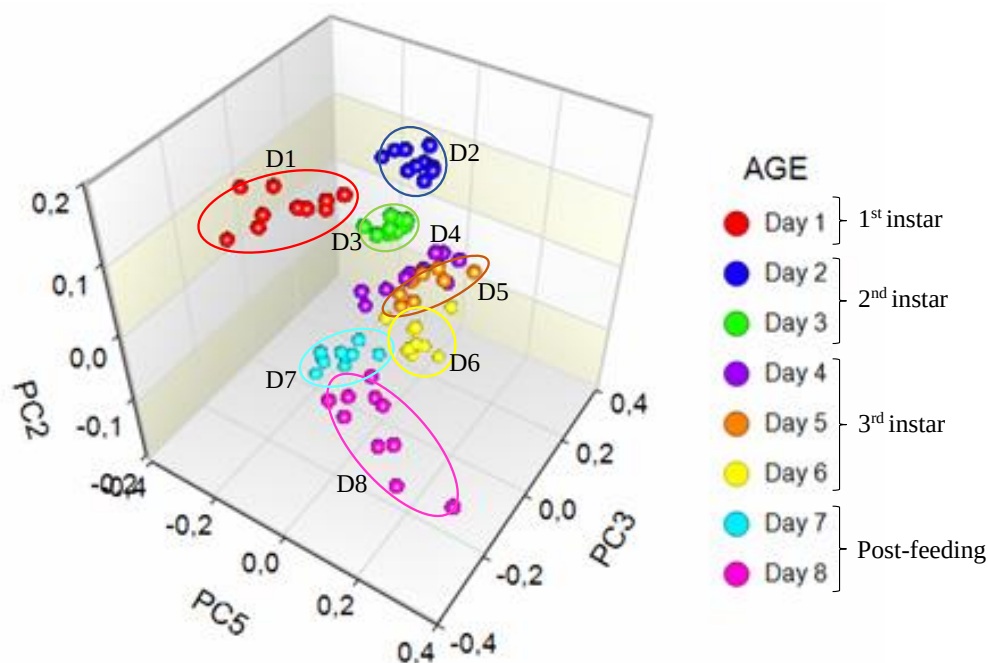


Figure 5.12: PCA 3D plot showing PC2 vs PC5 vs PC3 for *L. sericata* larvae reared indoors, using n-alkanes and branched methyl alkanes, with clustering days circled

The chemical changes between clusters could be traced, and there was still a systematic direction within the days, which moved clockwise from top to bottom. In addition, larval development stages were also distinctly visible among clusters. Day 1 cluster representing the first instar larvae was positioned at the top, while Days 2 and 3 were located just after it in the second instar larval stage. Days 4, 5 and 6 appeared in the third instar stage in the middle. Since these clusters contained small chemical changes in chemical profiles, they were located close to each other. The larvae in the post-feeding stage were located at the bottom on Days 7 and 8. The *L. sericata* larvae reared under constant temperature were aged to the day thanks to the 3D plot obtained using the CHC analysis and PCA.

5.2.2 Discussion of Ageing Larvae

The temperature has a significant influence on the development of the larvae and the decomposition process [52,149]. Until now, constant temperatures have been used in age estimation by CHC analysis of Diptera species. Sharma et al. [80] aged the developmental

stages of *Ch. rufifacies* larvae taken from laboratory stock and reared them under constant temperature using CHC analysis. Moore [40] reared *L. sericata* and *C. vicina* larvae under a constant temperature ($24\pm 1^{\circ}\text{C}$) and examined the age-related changes in their CHC profiles. These studies showed that CHC analysis could potentially be used to determine immature larval age and thus aid in PMI_{min} estimations. However, it has been stated that future studies are needed to confirm these results under natural conditions in the field. It is not known how the variables of the chemical profile are under fluctuating temperature. Therefore, taking Moore's [40] work as a premise, the changes in the CHC profiles of *L. sericata* and *C. vicina* larvae reared outdoors in the natural environment and a constant temperature of $25\pm 1^{\circ}\text{C}$ (indoors) were investigated. The effect of fluctuating temperature on the CHC profiles was investigated by comparing the changes in CHC profiles of larvae reared outdoors and indoors.

When discovering a cadaver at the crime scene, the temperature is often unknown and taken from the nearest meteorology station. However, there may be differences between the meteorological data and the temperature data of the crime scene due to environmental factors [150]. Therefore, in this study, the examination was based solely on ambient weather temperature during the development periods recorded to the day with an individual datalogger. These data are given in Appendices 1 to 6.

According to research on *A. grahami* larvae [146], fluctuating temperature results in a longer development time and slower growth rate than a constant temperature. Alotaibi et al. [147] examined the size and CHC profiles of *Chrysomya albiceps* larvae reared at temperatures ranging from 30°C to 45°C and evaluated their utility as an age indicator in different larval stages and the influence of temperature. The results of the current study revealed that the duration of the larval stage gradually decreased, whereas larval body size steadily increased throughout larval development as the rearing temperature increased. It was seen that both the larval development period and the larval body size are temperature dependent. Regarding CHC profiles, a significant difference was detected between different larval stages reared at the same temperature and between the same larval stage reared at different temperatures. Furthermore, it was found that the quantity of CHC identified in the profiles altered with temperature. However, data obtained only

at constant temperatures to predict PMI_{min} is unreliable, as the probability of encountering constant temperature in an authentic forensic case is low.

The temperature effect was clearly determined even by observing the percentage concentrations of CHC compounds detected in *C. vicina* and *L. sericata*. Larvae exposed to fluctuating temperatures in the field accumulated more CHC compounds in the chemical profiles to protect their living organism against temperature changes than the larvae reared under constant temperature. Current study results, including fluctuated temperatures, give a valuable benchmark for calculating PMI_{min} at an outdoor crime scene.

In the early immature stage, short-chain length CHCs provide more mobility and flexibility to the larvae. In order to increase the flexibility of the cuticle, branched methyl alkanes and alkenes with lower melting points are needed. As the larvae grow and develop, especially in the post-feeding stage, the cuticle mobility and flexibility decrease and longer chain CHCs are needed [39]. The increase in long-chain CHC in post-feeding larvae and pupae seeking dry habitat reflects the need for improved waterproofing. When the larvae get older and gradually move into the post-feeding stage, they move away from the food source and look for a place for the pupation stage. Therefore, they need extra waterproofing properties. In the earlier stages, they are submerged in food sources because of the warm and humid environment.

Larvae reared at a constant temperature and grew in size as they developed from the first instar to the third instar larvae. In this case, an increase in total CHC concentrations is expected. At the end of the third instar stage, the larval size remains constant for a while. When the larvae enter the post-feeding stage, they go into digestion and begin to prepare for puparia. The size of the larvae starts to shorten. However, the number of CHCs with a higher carbon number increases to the puparia, a solid chemical profile. Thus, the organism aims to protect itself against external environmental conditions and water loss at the puparial stage. Since CHCs with higher carbon numbers have higher boiling points, they protect against water evaporation more effectively. Again, the increase in the number of branched methyl alkanes is an expected change since the branching in the chemical profiles directly affects the boiling point.

Previous studies [24,147] reported that short-chain CHCs with lower boiling points were abundant in the first days of the larval stage, while long-chain CHCs with higher boiling points increased in the post-feeding stage. Zhu et al. [22] determined the larval age of *Ch. rufifacies* from one to eight days old using CHC analysis. They found that CHC profiles of larvae changed significantly with age. The statistical results showed that simple peak ratios of C29:H increased significantly with age. The results showed that the relative concentration of short-chain CHCs was greater in immature larvae than in older larvae. These prior findings are in line with those made using *C. vicina* and *L. sericata* larvae. Consequently, it is concluded that the composition of CHCs in insect species varies according to the physical needs of the larvae at each developmental life stage. Thus, CHC analysis of larvae has excellent potential for age estimation.

In the CHC profiles of *C. vicina* and *L. sericata* larvae reared under constant temperature, as expected, especially for the first and second instar larvae, short-chain length CHCs containing predominantly 20 to 25 carbons (linear or branched at positions 9, 7, 5, 4, 3) were found. In contrast, in post-feeding larvae, there was an increase in the relative concentration of long-chain length CHCs containing 26 to 31 carbons (linear or branched at positions 13, 11, 9, 7, 5, 4, 3). Furthermore, as the larvae aged, differences were manifest in the relative concentrations of CHC components for both species from the first larval day to the pupae stage.

Moore [40] showed that although the number of n-alkanes throughout the larval life cycle remained reasonably consistent, there were differences in the percentage concentration of each n-alkane with long carbon chain odd-chain n-alkanes (C29:H, C31:H and C33:H). It also showed that the relative concentrations of those n-alkanes increased with age, while n-alkanes with short and medium-chain length gradually decreased with age. These changes were provided to ageing the larvae to the day by PCA. Moore examined in detail all profiles of larvae under constant temperature. Since the part of the current study, results were not obtained under constant temperature; it was impossible to follow a regular decrease or increase in the CHC profiles of the sample sets of *C. vicina* and *L. sericata* larvae reared in the field. Moore's findings are supported by the results of the second half of the experiment, which was conducted at a constant temperature. When the chromatograms of the larval extracts were examined, it was observed that short or long-

chain CHCs appeared or disappeared under fluctuating temperature, contrary to what was observed under constant temperature and expected to change in the same larval stages.

Temperature, especially temperature differences between day and night, strongly influences the cuticle layer [26]. When the temperature data (Appendix 1 and 4) were examined, sudden decreases or increases in temperatures due to the weather or day and night were reflected in contemporaneous changes in the chemical profiles of cuticle layers. Larvae undertake countermeasures to protect themselves when exposed to temperature changes and environmental conditions such as rain and wind; therefore, they change their CHC profiles to minimize their exposure to these temperature changes [98,151].

When the CHC profiles obtained from both the control group and the outdoors-reared larvae were compared, it was observed that the larvae reared at variable temperatures contained more CHC compounds in both number and concentration than the larvae reared at a constant temperature.

Although some of the CHC compounds detected in chromatograms are mentioned as age indicators, this was insufficient for day-level ageing for the entire larval development stage. On the other hand, PCA provides a fast and straightforward age estimation to the day by visualising differences in profiles. In this study, PCA was used to follow the changes in CHC profiles and visualise the results over age and estimate larval age and track the differences between age clusters.

When the obtained scores were used in a 2-axis plane, it was noticed that the distribution between the clusters was not precise. A third score was added to the PC2 and PC3 scores to create a three-axis plot. Although Moore [40] provided an exact age estimation for the same larvae species, the current study used 3D plotting for age-specific estimation, especially for the day level, for almost all larval age clusters.

Different larvae species collected outdoors may look highly similar and grow to different body lengths, and reliable age estimation is difficult due to the variable impacts of temperature. The ageing of post-feeding larvae is difficult due to larval size shortening and swelling for pupation preparation, and the duration of the stage takes more than one

day. Therefore, previous physiological and morphological investigations failed to provide a reliable age indication to the day. However, in this method combining CHC analysis and PCA, age estimations of post-feeding larvae could be made to the day. All these studies support that CHC analysis can be used for age estimates of *L. sericata* and *C. vicina* larvae species and the stability of the CHC profile is sufficient to provide age estimation even for authentic wild samples.

5.3 Puparial Stage

Larvae are the most commonly used evidence for ageing, but if the pupae stage has already begun at a crime scene, the larval age estimation cannot be used. In this case, the only useable evidence for age estimation is puparia. Furthermore, the puparial stage is critical, as it constitutes 50% of the immature development cycle. It is like a sealed box in which metamorphosis occurs. Although only minor changes in their outer shell are visible from the outside, like colour, the tissues and organs undergo a significant internal regeneration (histolysis and histogenesis) [17]. The pupae are composed mainly of body fluid at some development stages of the puparia. The larval fat and glycogen stores are used to develop pupae or adult fly chemical profiles, such as the flight muscle and digestive system [65].

Metamorphosis is not visible from the outside, and there are no distinct stages in puparial development, which makes it difficult to track. Nevertheless, specific characteristics, such as the production of setae or setal colouration, can be regarded as discrete developmental signals on coarse time scales and as continuous development processes on the finer temporal resolution [152], cellular proliferation, tissue remodelling, cell migration, and programmed cell death [141]. In the pupae stage, development can be followed by gene expression [20], histological methods [65], hyper chromatogram imaging [136], micro-computed tomography [7] and X-ray video imaging [153]. However, these methods are not preferred, as they require advanced level experience; they are laborious, expensive and time-consuming. Also, even though age estimation methods are generally focused on the larval stage, there is little published data for age estimation of pupae in the literature. On the other hand, using CHC analysis, the chemical profile required can be easily extracted, and qualitative and quantitative results can be obtained with this method.

Therefore, the CHC analysis method is a practical application for forensic entomology [77].

Like the larvae cuticle, the pupae wall contains various hydrophobic lipids, including long-chain n-alkanes, branched methyl alkanes, alkenes and aliphatic waxes [154]. The aim is to create a hydrophobic layer that protects the living being from dehydration and the penetration of microorganisms.

The relative concentrations and specific compounds of the CHCs layer change with age, diet and temperature conditions in the pupae stage, like in other life stages. Zhu et al. [22] stated that increased concentration of long-chain CHCs with age may be due to the dry and advanced waterproofing requirement of the larvae and adult flies at the pupae stage. Frederickx et al. [84] examined changes in volatile compounds released by the larvae and pupae of *C. vicina* to the day by headspace solid-phase microextraction followed by GC-MS. Roux et al. [145] examined the entire developmental ontogeny of CHCs in three adult fly species, *C. vomitoria*, *C. vicina* and *P. terraenovae*. Their studies at the pupae stage also stated that it was difficult to distinguish after the first few days due to the stability of the CHC profile. Again, Moore [37] mentioned the difficulties in age estimation of *L. sericata*, *C. vicina* and *C. vomitoria* pupae using CHC analysis. These studies were carried out under constant temperatures, and it is unknown whether the CHC components of the pupae reared outdoors can be used for age estimation and how their stability changes under fluctuating temperatures.

This section of the chapter presents the age estimations of *C. vicina* and *L. sericata* pupae species using CHC analysis and PCA. Pupae of both species were reared outdoors. Furthermore, control groups were formed in the 25±1°C constant temperature incubator for both species. It aimed to age the pupae reared outdoors with the CHC analysis and examined the effect of fluctuating temperature on the CHC profiles of the pupae species.

5.3.1 Results for Puparia

5.3.1.1 *C. vicina* puparia

5.3.1.1.1 *Fieldwork study*

Since day-level CHC differentiation in the puparial stage is very limited, taking samples every other day during the pupation stage extractions was the preferred strategy adopted in this study. The adult flies hatch out of the pupae on Day 12; thus, the last extractable puparial day was Day 11. In the GC chromatograms of the puparia, 39 CHC compounds and peaks of unidentified hydrophobic oils were detected. The chain lengths of the CHC compounds were between C16:H and C33:H. The total CHC concentrations of CHC profiles during the puparial stage were as follows: n-alkanes (37%), alkenes (7%) and branched methyl alkanes (56%). Although the number of branched methyl alkanes was higher than n-alkanes, the predominant group in concentration was n-alkanes. Table 5.5 shows the identified CHCs extracted from the pupation and the total percentage of each compound, and the percentage standard deviation values from Day 1 to Day 11.

Table 5.5: CHC compounds extracted from *C. vicina* pupae reared outdoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 3	DAY 5	DAY 7	DAY 9	DAY 11
1	C16	1.79±0.41	0.55±0.16	0.84±0.41	1.40±0.49	tr	1.02±0.22
2	C17	tr	tr	0.52±0.17	0.61±0.20	tr	0.51±0.12
3	7-MeC17	tr	0.51±0.09	tr	tr	tr	tr
4	3-MeC17	1.40±0.18	1.64±0.40	1.44±0.18	1.46±0.19	0.91±0.14	0.99±0.13
5	Octadecene*	0.57±0.10	0.55±0.17	0.63±0.10	0.66±0.09	0.50±0.09	0.55±0.07
6	C18	3.07±0.37	3.34±0.88	3.38±0.42	3.33±0.43	2.28±0.33	2.96±0.33
7	5-MeC18	tr	tr	tr	3.62±0.92	tr	tr
8	3-MeC18	1.60±0.18	2.12±0.37	1.91±0.24	1.78±0.22	1.24±0.20	1.30±0.27
9	Eicosene*	tr	0.53±0.17	0.60±0.12	0.54±0.09	0.50±0.09	0.55±0.21
10	C20	2.62±0.30	3.26±0.95	3.21±0.38	2.93±0.39	2.45±0.42	2.71±0.72
11	3-MeC20	1.72±0.28	2.46±0.94	2.50±0.92	1.88±0.59	1.80±0.67	1.87±0.75
12	Docosene*	0.81±0.12	0.58±0.23	2.72±1.95	1.55±0.55	tr	2.55±0.70
13	C22	1.94±0.24	2.35±0.72	3.00±1.20	2.34±0.75	2.66±1.10	2.97±1.05
14	C23	tr	tr	tr	0.55±0.49	0.61±0.39	0.55±0.42
15	4-MeC23	tr	1.00±0.45	tr	tr	tr	tr
16	3-MeC23	1.14±0.14	1.71±0.52	1.47±0.20	1.34±0.17	1.13±0.24	1.33±0.30
17	C24	1.22±0.15	1.69±0.52	1.24±0.18	1.16±0.15	1.03±0.32	1.39±0.36
18	C25	49.95±8.94	36.57±12.74	42.74±7.55	37.43±10.15	42.03±7.17	33.89±7.35
19	11+13-MeC25	tr	0.54±0.15	tr	tr	tr	0.55±0.16
20	7-MeC25	tr	0.50±0.08	tr	tr	tr	tr

Peak No.	Peak ID	DAY 1	DAY 3	DAY 5	DAY 7	DAY 9	DAY 11
21	3-MeC25	1.08±0.16	1.60±0.48	1.34±0.24	1.23±0.16	1.05±0.18	1.44±0.30
22	C26	2.43±1.09	1.74±0.49	1.74±0.32	1.67±0.28	1.62±0.28	2.06±0.50
23	C27	10.22±2.52	12.31±3.58	12.89±4.34	13.44±2.84	15.37±3.69	15.05±4.72
24	13+15-MeC27	tr	0.87±0.32	tr	0.58±0.15	tr	tr
25	11+13-MeC27	tr	0.95±0.50	0.86±0.48	0.51±0.09	0.68±0.14	0.89±0.20
26	3-MeC27	1.59±0.39	2.32±0.69	2.03±0.63	1.93±0.41	2.13±0.48	2.69±0.72
27	C28	1.43±0.26	1.71±0.43	1.69±0.49	1.73±0.31	1.74±0.38	1.74±0.42
28	4-MeC28	tr	tr	tr	tr	0.55±0.11	0.51±0.15
29	3-MeC28	tr	tr	tr	0.82±0.25	0.84±0.27	0.87±0.25
30	C29	6.31±1.31	8.35±2.39	7.75±2.12	8.53±1.62	9.14±2.17	7.93±1.75
31	3-MeC29	0.67±0.18	tr	0.72±0.24	0.64±0.10	0.63±0.20	0.85±0.16
32	C30	0.61±0.24	0.68±0.28	0.69±0.21	0.58±0.09	0.54±0.13	0.63±0.15
33	8-MeC30	tr	tr	tr	tr	tr	0.56±0.27
34	4-MeC30	tr	tr	tr	tr	tr	0.52±0.11
35	C31	2.83±0.75	3.11±1.08	3.84±0.73	3.45±0.60	3.08±0.55	2.81±0.62
36	13+15-MeC31	1.63±0.45	1.09±0.53	tr	tr	1.08±0.39	1.34±0.42
37	3-MeC31	1.11±0.38	1.18±0.43	tr	tr	0.69±0.24	0.71±0.20
38	C32	1.17±0.44	1.24±0.37	tr	tr	1.43±0.25	1.33±0.36
39	C33	1.09±0.44	1.45±0.54	tr	1.39±0.43	1.05±0.28	1.37±0.63

tr = Trace amount detected (<0.5%)

* Double bond positions were not determined

In Figure 5.13, the chromatograms of the *C. vicina* pupation period are given as Days 1, 3, 5, 7, 9 and 11. The marked areas show the peaks of n-alkanes whose concentration changed significantly with age. The presence of C18:H to C31:H n-alkanes in the CHC profiles was identified throughout the puparial period. Except for the changes in the percentage concentrations of C25:H, C27:H and C29:H n-alkanes, there was almost no change in the percentage concentrations of other n-alkanes. The decrease in the percentage concentration of C25:H n-alkanes and the increases in C27:H and C29:H concentrations were the most noticeable changes in the chemical profiles with age.

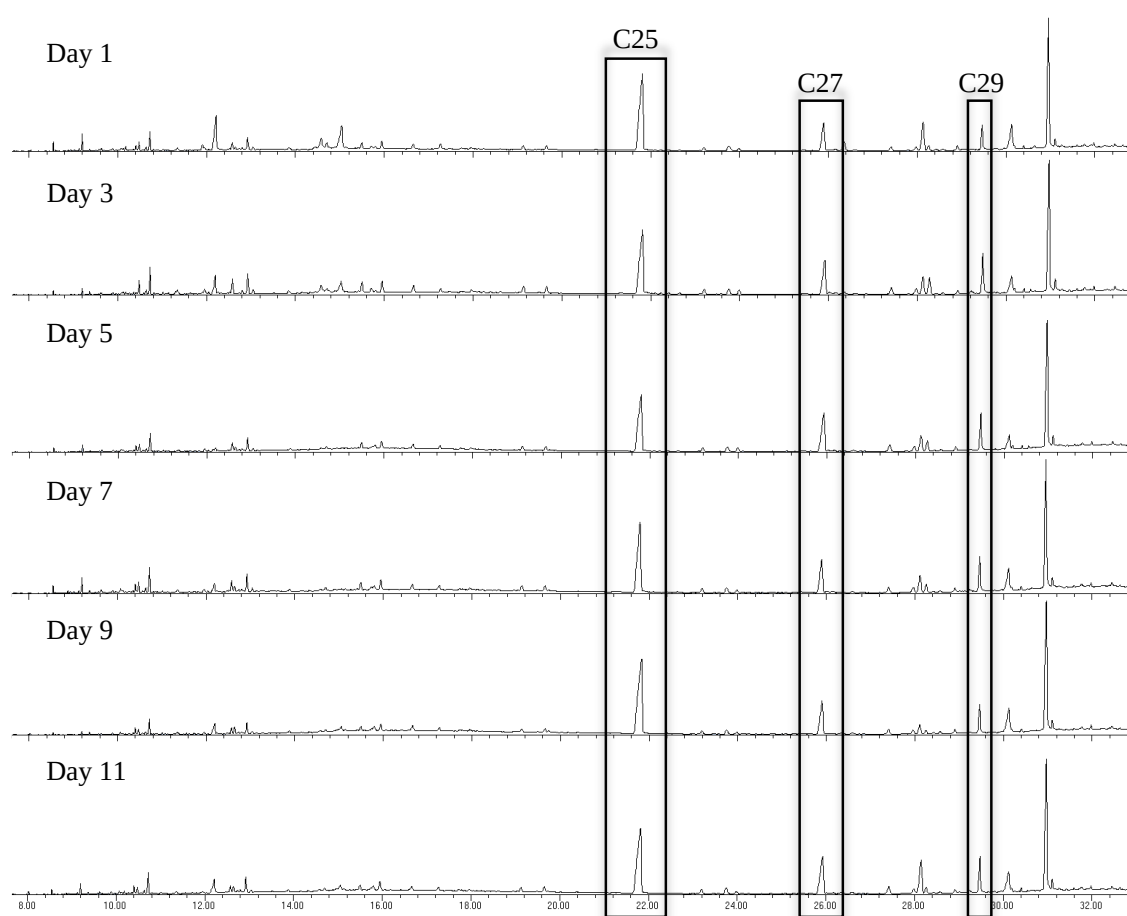


Figure 5.13: GC chromatograms of *C. vicina* pupae reared outdoors for Days 1, 3, 5, 7, 9 and 11, respectively

However, the change in the percentage concentrations of n-alkanes alone was not sufficient to distinguish between clusters. The variation of the percentage concentration of branched alkanes (Appendix 15), their existence, or absences in the chemical profiles with age was taken as the basis for puparial ageing. Branched methyl alkanes with greater percentage concentrations were 3-MeC17, 3-MeC18, 3-MeC20, 3-MeC23, 3-MeC25 and 3-MeC27. The percentage concentration of these compounds increased from Day 1 to Day 3 and then decreased until Day 9. There was an increase again on Day 11. Although there was no specific compound detected only on Day 1, the absence of the 11+13-MeC27 compound, which was detected at other puparial ages, was the specific age indicator for Day 1. 7-MeC17 and 4-MeC23 branched methyl alkanes were detected only on Day 3, for which they were age indicators. 7-MeC17 was detected with higher concentration only on Day 7, also as an age indicator. The 8-MeC30 and 4-MeC30 isomers detected in Day 11 pupae were also specific for this age.

PCA was applied to the data obtained to visualise the differences between puparial ages and to determine ages. All n-alkanes, alkenes and branched methyl alkanes, defined in Table 5.5, were applied for the PCA calculations. The PCA plot of *C. vicina* pupae reared outdoors is shown in Appendix 16. With this PCA plot, it was not easy to distinguish the age clusters representing pupation time, although there was a relative separation. The distribution between age clusters was not sufficient. The clusters were scattered and overlapping. The most stable CHCs in the chemical profiles of pupae were n-alkanes, including C25:H, C27:H and C29:H, and the age-related variation in their concentrations was minimal. Therefore, PCA was performed on additional data set by removing the n-alkanes. The new PCA plot for *C. vicina* pupae reared outdoors using a second data set containing only alkenes and branched methyl alkanes is shown in Appendix 17, providing a more apparent separation between the age clusters. On the 2D plot, the clusters were not scattered except for the Day 5 cluster. Although a good separation was obtained on this plot prepared using PC3 against PC2 scores, Days 3 and 9 almost overlapped. Thus, the 3D plot (Figure 5.14) was formed to obtain a more precise separation.

As used for the larval period, a 3D PCA plot (Figure 5.14) was drawn using the PC2, PC3 and PC5 scores obtained by PCA. PCA was performed using six main components explaining 88.0% of the data set variation, with the first three principal components being 53.5, 22.6 and 11.9%, respectively.

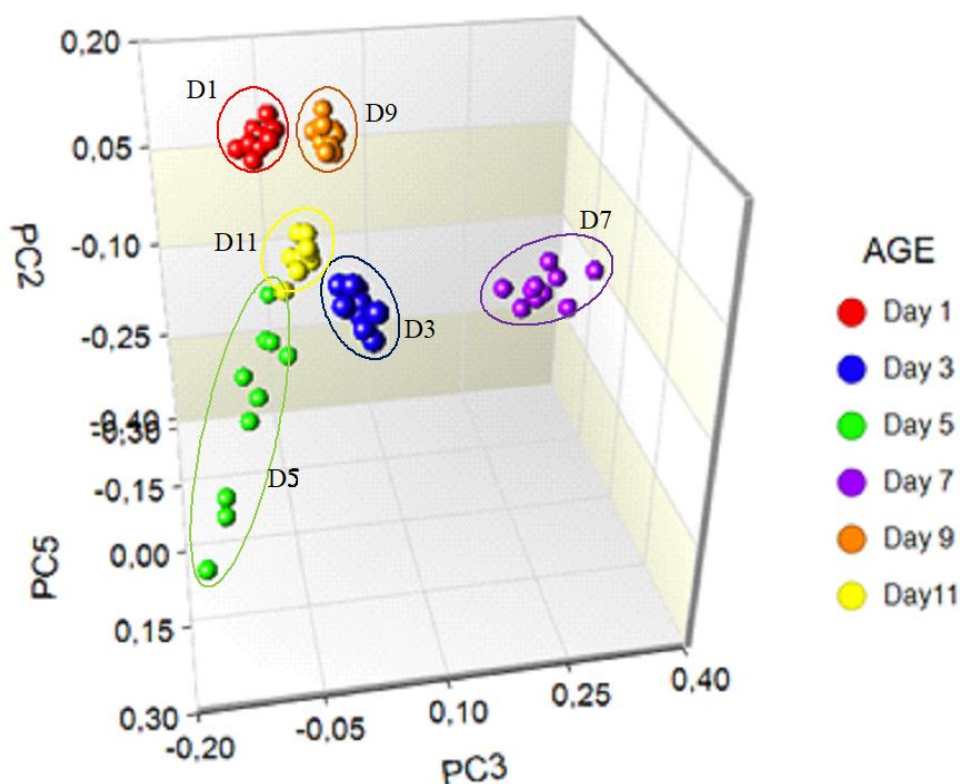


Figure 5.14: PCA 3D plot showing PC2 vs PC5 vs PC3 for *C. vicina* pupae reared outdoors, using alkenes and branched methyl alkanes only, with clustering days circled

With a third axis to the 2D dataset, the seemingly overlapped Day 3 and Day 9 clusters were separated clearly; the separation of age clusters was apparent. Other than Day 5, the other age clusters were not scattered. The *C. vicina* pupae reared outdoors were separated with CHC analysis, and age estimations were successfully performed to the day with PCA. Obtaining this level of age estimate for both outdoor-rearing and the pupae stage is a very promising basis for future research.

5.3.1.1.2 Control group at constant temperature (25±1°C)

To compare the effect of temperature on the CHC profile, a control group reared under a constant temperature of 25±1°C was established. The *C. vicina* pupae reared at a constant temperature were extracted at one-day intervals until they metamorphosed into adult flies. In this section, the chemical changes occurring in the chemical developing indoors and outdoors were comparatively analysed to determine the effect of temperature on the puparium and to estimate the age of the pupae reared indoors.

The total percentages of the CHC profile of *C. vicina* pupae contained 11 identifiable CHC compounds, given in Table 5.6, consisting mainly of n-alkanes (64%) and branched methyl alkanes (46%) during the puparial period. Since the adult flies hatch out of the pupae on Day 10, the last extractable puparial day was Day 9. The carbon chain lengths of the CHC compounds were between C21:H and C31:H. The predominant group in CHC profiles are n-alkanes in terms of total concentration and number. Most of the more volatile compounds that appeared within 8 to 16 minutes were composed of other non-polar compounds and were therefore excluded from the CHC identification.

Table 5.6: CHC compounds extracted from *C. vicina* pupae reared indoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 3	DAY 5	DAY 7	DAY 9
1	3-MeC21	23.10±8.81	tr	15.94±6.31	11.21±6.40	tr
2	C25	tr	tr	5.35±1.63	6.12±2.95	5.25±1.87
3	C26	tr	tr	tr	tr	5.62±1.90
4	C27	25.91±13.52	33.60±9.38	31.18±16.76	34.65±24.08	37.34±13.97
5	3-MeC27	tr	8.30±4.08	4.07±2.26	7.59±5.59	6.52±3.52
6	C28	6.80±3.34	6.47±1.78	5.79±2.91	8.43±4.99	7.53±4.65
7	C29	35.59±11.19	33.41±10.08	29.35±12.00	27.77±11.16	30.43±9.91
8	13+15-MeC29	tr	8.15±3.45	2.55±1.52	3.67±2.32	tr
9	3-MeC29	tr	tr	tr	tr	1.89±1.52
10	C30	2.23±0.64	tr	2.00±1.10	1.89±1.02	1.50±0.49
11	C31	6.35±2.71	5.26±2.03	3.76±0.96	4.78±2.31	3.92±1.21

tr = Trace amount detected (<0.5%)

In Figure 5.15, GC chromatograms of *C. vicina* pupae reared outdoors and indoors are given for Day 1 and the last pupation day before the adult fly stage. The volatile and CHC compounds were higher in CHC profiles reared outdoors than in pupae reared indoors. C25:H and C27:H concentrations in pupae reared outdoors were much higher than in pupae reared indoors. While the C29:H concentrations were almost similar, the C30:H concentrations were also relatively higher than the pupae reared indoors.

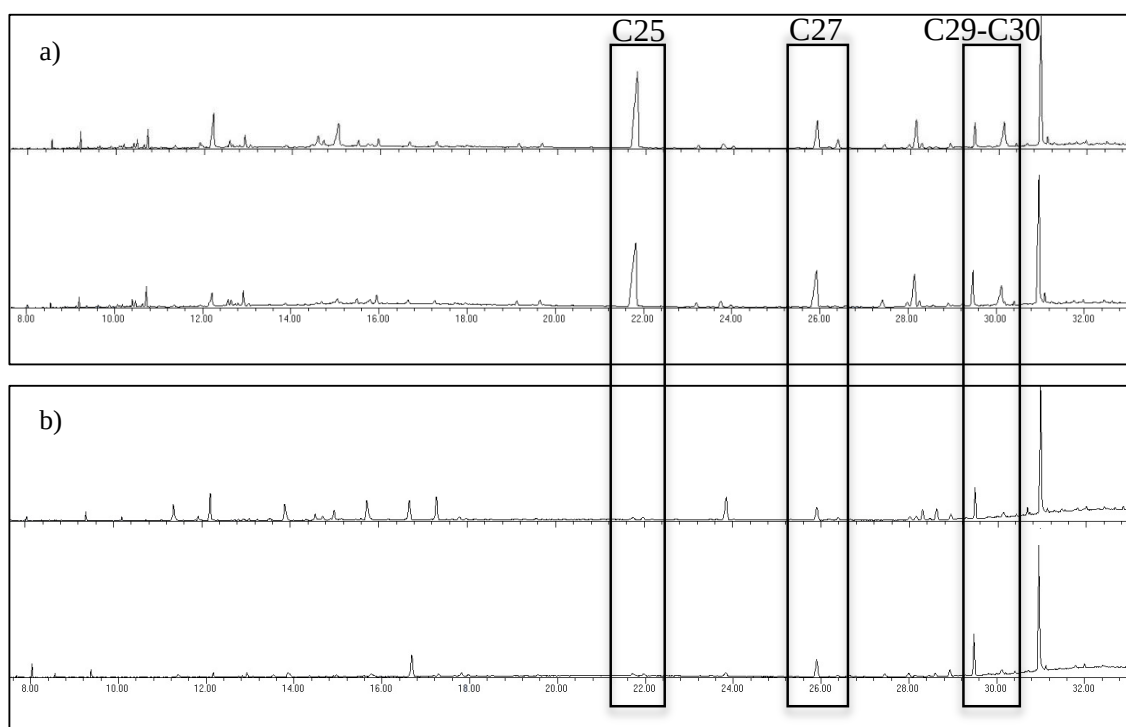


Figure 5.15: GC chromatograms of *C. vicina* pupae for Day 1 and last pupation day before the eclosion, respectively, reared a) outdoors b) indoors

Observing the change in the percentage concentrations of total n-alkanes and branched methyl alkanes reared outdoors and indoors obtained extractions on Day 1 of the pupae and the day just before the emergence of adult flies (Appendix 18), the n-alkane percentage concentration in the *C. vicina* pupae reared outdoors decreased from the first day to the last day of the pupation, while the branched methyl alkane concentration increased. Conversely, for *C. vicina* pupae reared indoors, the n-alkane total concentration increased from Day 1 to the last puparial day, while the branched methyl alkane

concentration decreased. There was an 8% decrease in the total n-alkane concentration and a 6% increase in the total methyl alkane concentration between the first day and the last pupation day reared outdoors. There was a 15% increase in the total n-alkane concentration for pupae reared indoors and a 15% decrease in the total methyl alkane concentration.

When observing the total percentage concentration of n-alkanes and branched methyl alkanes extracted from pupae reared outdoors and indoors, it was seen that the pupae reared outdoors had as much branched methyl alkane percentage concentration as the pupae reared indoors. In contrast, the percentage concentration of n-alkanes in outdoor pupae was much higher than indoor pupae. With this plot, the inference from the chromatograms was quantitatively matched with CHCs.

In addition to examining the effects of indoor and outdoor rearing on the CHC profiles of puparia, the ageing of pupae was also included in this study. In order to obtain more distinct age clusters, as in pupae reared outdoors, the 3D PCA plot (Figure 5.16) was created by applying branched methyl alkanes of *C. vicina* puparium. In order to obtain a clear separation between clusters, n-alkanes were eliminated from the dataset of Day 1 to Day 9. PC2, PC3 and PC4 scores were used to generate the three axes of the plot. PCA was performed using six main components explaining 96.3% of the data set variation, with the first three principal components being 65.3, 24.8 and 6.2%, respectively.

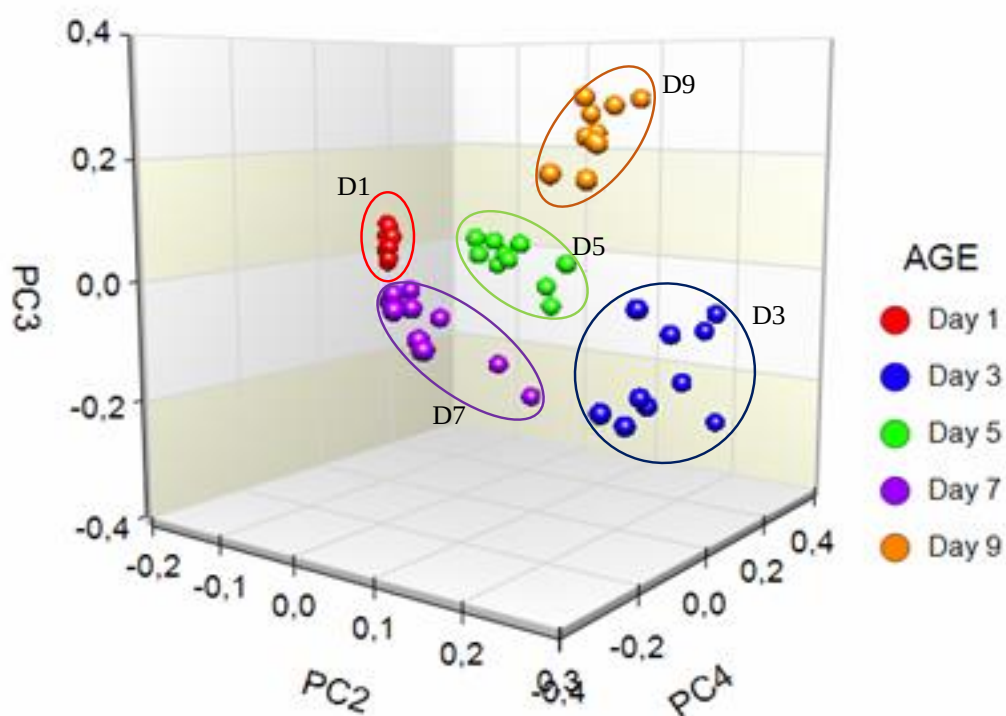


Figure 5.16: PCA 3D plot showing PC3 vs PC2 vs PC4 for *C. vicina* pupae reared indoors, using branched methyl alkanes only, with clustering days circled

The separation between age clusters was distinct in the plot, although the repetitive patterns that make up the age clusters showed scattered within themselves. The separation of Day 1 and Day 9 clusters shows the chemical change in the puparial chemical profiles at the beginning and end of the puparial stage. The *C. vicina* pupae reared indoors were aged to the day thanks to the 3D plot obtained using the CHC analysis and PCA. Since the temperature effect was minimized as the constant temperature was used, it was easier to follow the changes between the cluster and estimate the age of puparia.

5.3.1.2 *L. sericata* puparia

5.3.1.2.1 Fieldwork study

The CHC profiles of *L. sericata* pupae reared outdoors were extracted at one-day intervals until they metamorphosed into the adult fly stage. As adult flies hatch from the pupae on Day 10, the last extractable puparial day is Day 9. In the GC chromatograms of the

puparia, peaks of the CHC compound and unidentified hydrophobic oils were detected. The chain lengths of the CHC compounds were between C20:H and C33:H. The CHC profiles consisted of n-alkanes (70%), alkenes (12%) and branched methyl alkanes (18%). The CHCs extracted and identified from the pupae (Table 5.7) were used in PCA calculations for the age estimation. The predominant group in CHC profiles are n-alkanes in terms of total concentration and number.

Table 5.7: CHC compounds extracted from *L. sericata* pupae reared outdoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 3	DAY 5	DAY 7	DAY 9
1	Eicosene*	tr	tr	tr	0.81±0.15	0.66±0.06
2	C20	tr	tr	tr	0.53±0.09	0.50±0.08
3	3-MeC21	tr	1.79±0.97	tr	tr	tr
4	Docosene*	tr	tr	tr	tr	0.51±0.03
5	C22	tr	tr	tr	0.52±0.09	0.50±0.10
6	C25	7.38±1.50	6.18±1.63	6.00±0.88	5.80±0.96	5.50±0.60
7	C26	tr	2.71±1.26	0.67±0.17	0.63±0.17	0.81±0.30
8	C27	20.20±4.85	19.61±4.18	22.53±5.56	20.01±6.45	23.24±6.74
9	3-MeC27	tr	tr	tr	1.09±0.30	1.41±0.71
10	C28	3.86±0.70	3.62±0.64	3.67±0.70	3.58±0.86	3.77±0.81
11	C29	48.93±8.48	47.34±9.73	48.50±8.02	48.38±12.89	46.01±8.29
12	7-MeC29	0.58±0.23	tr	1.08±0.36	tr	0.57±0.20
13	C30	2.36±0.49	2.30±0.73	1.92±0.58	2.43±0.99	2.25±0.52
14	C31	14.11±3.18	13.70±3.66	13.15±2.45	13.44±3.79	11.94±2.21
15	C32	1.46±0.40	1.49±0.21	1.38±0.29	1.55±0.43	1.37±0.38
16	C33	1.11±0.25	1.26±0.45	1.10±0.32	1.09±0.37	0.97±0.24

tr = Trace amount detected (<0.5%)

* Double bond positions were not determined

In Figure 5.17, the chromatograms are given in sequential order from Day 1 to Day 9. As well as the presence of the compound in the chemical profile, absence can also be considered an indicator. C25:H, C27:H and C29:H n-alkanes were detected extensively in the CHC profiles of *L. sericata* puparia. The most predominant compound in chemical profiles was C29:H. There was little change even for the C25:H, C27:H and C29:H n-alkanes, where generally the most changes occurred. The chain lengths of the CHCs in the Day 1 age pupae varied between C25:H and C33:H. In this context, C26:H n-alkane, which cannot be detected only in Day 1 puparia, was a specific age indicator for this age. In Days 7 and 9 age puparia, chain lengths varied between C20:H to C22:H and could only be detected at these ages. The compound 3-MeC21 was detected only in the Day 3 samples and was indicative of this age. Since the 3-MeC27 compound was detected only on Day 7 and Day 9, it was an indicator for these ages. Docosene was detected only on Day 9 and was an age indicator for this age.

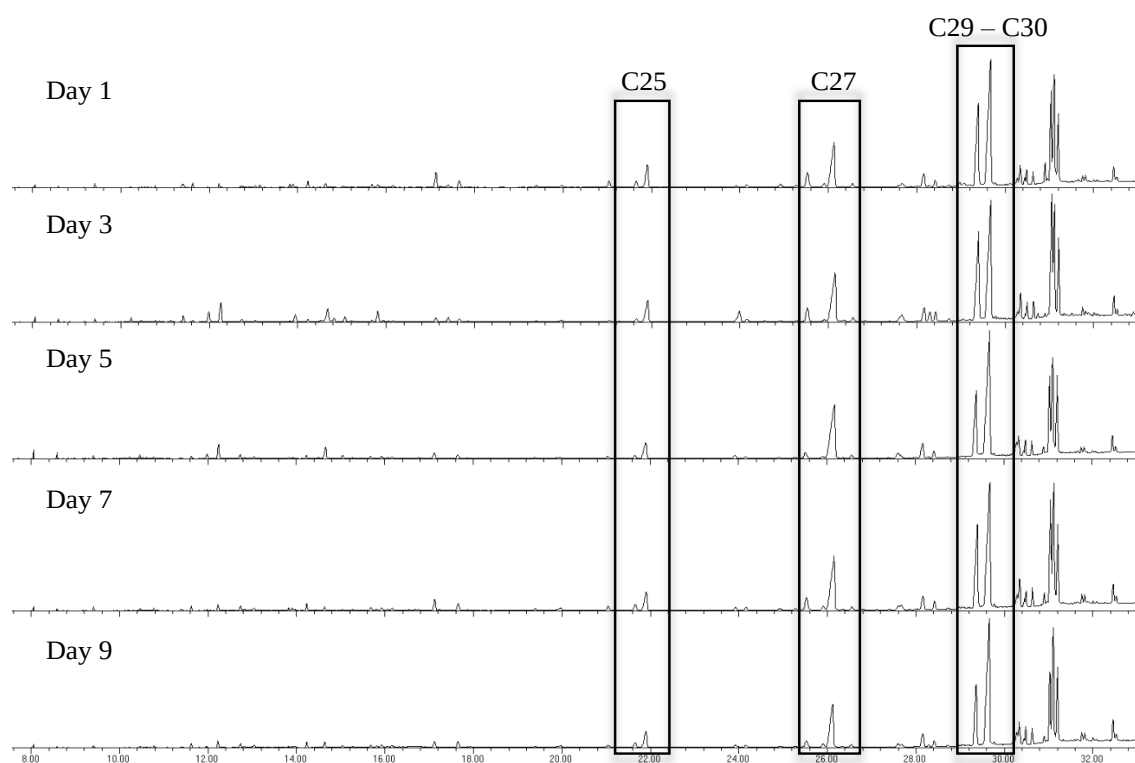


Figure 5.17: GC chromatograms of *L. sericata* pupae reared outdoors from Days 1, 3, 5, 7 and 9, respectively

n-alkanes were the most predominant CHCs in the chemical profiles of puparia, and the amount of change in their concentrations with age was low. Therefore, PCA was conducted on an extra data set after the n-alkanes had been removed from the original CHCs. For PCA, only data of branched methyl alkanes were applied in order to achieve well-separation. The 3D PCA plot of *L. sericata* pupae reared outdoors is given in Figure 5.18. PCA was performed using six main components explaining 98.8% of the data set variation, with the first three principal components being 89.9, 5.7 and 3.1%, respectively. PC2, PC3 and PC4 scores were preferred as they provided more distinct separation between clusters.

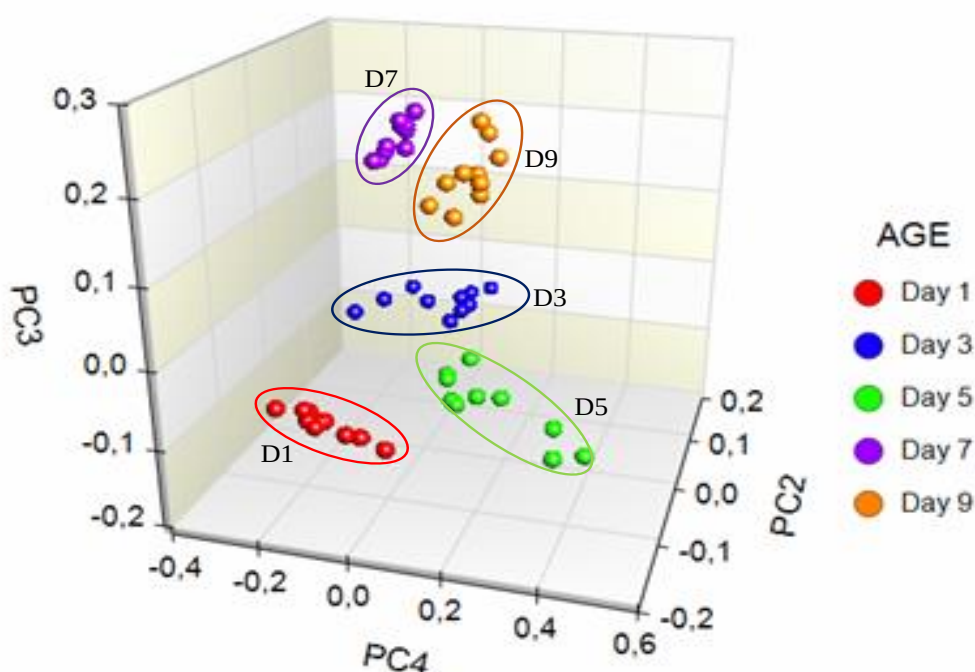


Figure 5.18: PCA 3D plot showing PC3 vs PC4 vs PC2 for *L. sericata* pupae reared outdoors, using alkenes and branched methyl alkanes only, with clustering days circled

The chromatograms of the pupae were very similar. Very few changes were obtained in the CHCs detected that could be used for quantitative and quantitative comparison as an age indicator. However, when the data obtained were visualized with PCA, a successful age estimation could be made between age clusters. All clusters were clearly separated

from each other. In particular, the separation of the clusters representing Day 1 and Day 9 puparial ages was necessary in terms of clearly showing the beginning and end of the puparial stage. With the CHC analysis and PCA, a highly accurate estimation of age was obtained for the *L. sericata* species reared outdoors for the puparial stage, where age estimation was complicated. The results presented identify promising avenues for future research directions.

5.3.1.2.2 Control group at constant temperature ($25\pm1^{\circ}\text{C}$)

In the CHC profile of *L. sericata* puparia, 10 identifiable CHC compounds were detected from Day 1 to Day 7. The total CHC concentrations of CHC profiles were n-alkanes (80%) and branched methyl alkanes (20%) (Table 5.8). The alkenes detected in insufficient concentrations ($<0.5\%$) were not included. The other non-polar compounds were excluded from the CHC identification. As adult flies hatched out on the eighth day, Day 7 was the last day of extraction of the puparial period. The CHCs chain lengths were between C25:H and C33:H. The predominant group in CHC profiles are n-alkanes in terms of total concentration and number.

Table 5.8: CHC compounds extracted from *L. sericata* larvae reared indoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 3	DAY 5	DAY 7
1	C25	50.65 $\pm$ 26.39	39.19 $\pm$ 9.72	39.58 $\pm$ 5.96	34.80 $\pm$ 9.42
2	C26	tr	2.12 $\pm$ 1.72	1.93 $\pm$ 1.09	3.15 $\pm$ 1.97
3	C27	4.27 $\pm$ 2.09	9.91 $\pm$ 3.55	11.00 $\pm$ 2.70	10.96 $\pm$ 3.42
4	3-MeC27	tr	1.50 $\pm$ 0.60	1.71 $\pm$ 0.44	1.62 $\pm$ 0.79
5	C28	1.66 $\pm$ 0.85	2.08 $\pm$ 0.72	2.12 $\pm$ 0.28	2.27 $\pm$ 0.85
6	C29	27.46 $\pm$ 12.93	28.79 $\pm$ 11.88	29.10 $\pm$ 3.27	31.55 $\pm$ 14.09
7	7-MeC29	tr	tr	tr	0.66 $\pm$ 0.26
8	C30	1.85 $\pm$ 0.89	1.95 $\pm$ 0.96	1.90 $\pm$ 0.40	1.84 $\pm$ 0.85
9	C31	12.74 $\pm$ 7.81	13.07 $\pm$ 6.01	11.38 $\pm$ 2.12	11.76 $\pm$ 5.06
10	C33	1.37 $\pm$ 0.84	1.38 $\pm$ 0.87	1.28 $\pm$ 0.42	1.39 $\pm$ 0.49

tr = Trace amount detected ($<0.5\%$)

In Figure 5.19, GC chromatograms of *L. sericata* pupae reared outdoors and indoors are given for Day 1 of pupation and the last pupation day before the adult fly stage. In the chromatograms, peaks with retention times of 12 and 16 minutes were generally polar compounds, so these polar compounds were not included. Peaks of compounds C25:H, C27:H and C29:H, with markedly varying concentrations in the CHC profile, were marked on chromatograms. The percentage concentration of C27:H, C29:H, and C31:H n-alkanes with higher boiling points were abundant in the pupae reared outdoors. In contrast, the percentage concentration of C25:H n-alkanes detected pupae reared indoors was higher than outdoors. The most predominant compound in indoor-reared was C29:H.

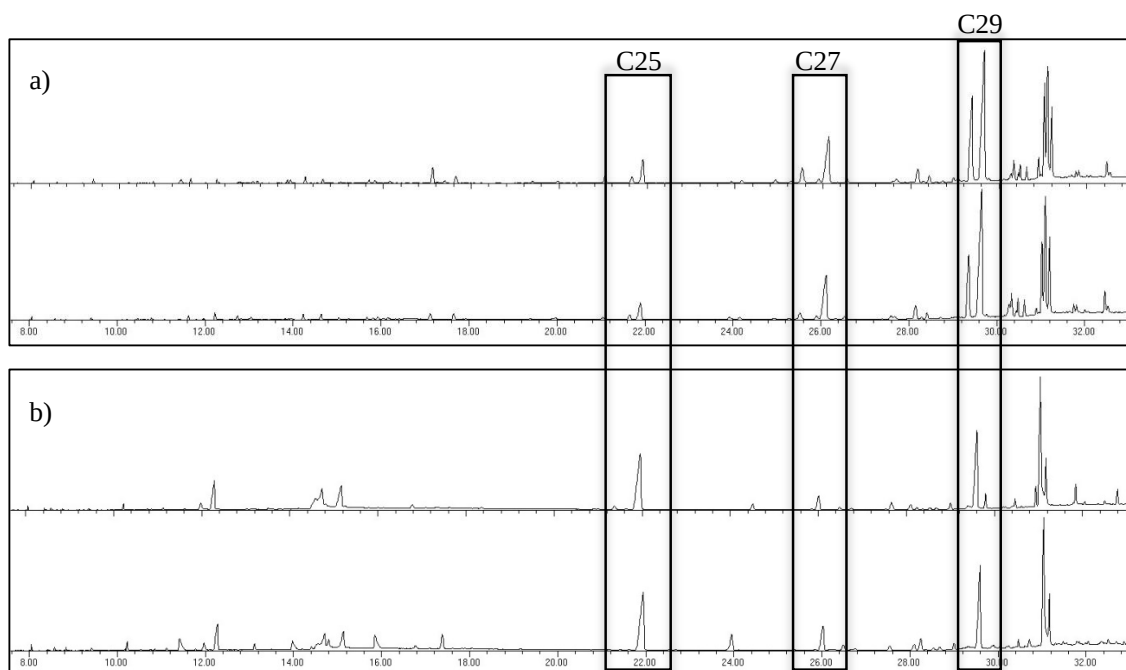


Figure 5.19: GC chromatograms of *L. sericata* pupae for Day 1 and last pupation day before the eclosion, respectively, reared a) outdoors b) indoors

When examining the results obtained from the extractions performed on the first day and the last puparial day just before the emergence of adult flies, there was a 6% increase in the total concentration of n-alkanes in the chemical profiles of pupae reared outdoors and a 14% increase in the total concentration of n-alkanes in the pupae reared indoors. During this process, the percentage concentration of branched methyl alkanes in the chemical

profiles outdoors increased by 59%; in contrast, no branched methyl alkanes could be detected in pupae reared indoors on the first day, but 3-MeC27 and 7-MeC29 branched methyl alkanes were detected on the last puparial day.

It was obtained that the pupae reared outdoors had as much branched methyl alkane percentage concentration as the pupae reared indoors. In contrast, the percentage concentration of n-alkanes in outdoors pupae was much higher than the indoor reared puparia. Since pupae reared outdoors were more affected by temperature, there were more n-alkanes in their chemical profile.

This study investigated the effect of indoor and outdoor rearing on the CHC profiles of *L. sericata* puparia and the ageing of pupae reared indoors in particular. Since the number of CHC detected in indoor-reared *L. sericata* pupae was already low, all detected compounds were used for PCA. PCA was performed using six main components explaining 99.5% of the data set variation, with the first three principal components being 83.6, 15.1 and 0.7%, respectively. PC2, PC3 and PC5 scores were used due to provide the most distinctive difference between age clusters. The 3D PCA plot of *L. sericata* puparium reared at $25\pm 1^{\circ}\text{C}$ is given in Figure 5.20.

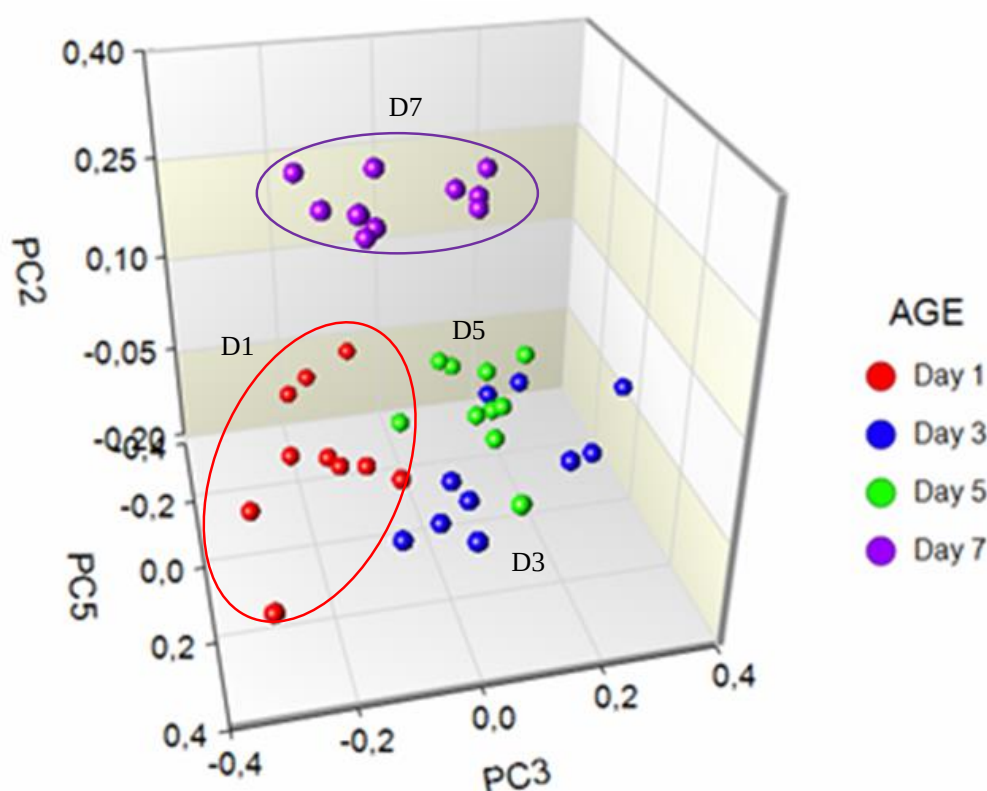


Figure 5.20: PCA 3D plot showing PC2 vs PC5 vs PC3 for *L. sericata* pupae reared indoors, using n-alkanes and branched methyl alkanes, with clustering days circled

On the PCA plot, the clusters representing Day 1 and Day 7, when the puparial stage started and ended, were distinctly separated from each other. Although the Day 3 and Day 5 clusters appear to overlap, they could be separated in PC5 loading. Furthermore, both developmental stages have similar chemical changes. This view of the plot was preferred because it aimed to highlight the age estimation between Day 1 and Day 7 clusters. With the results presented, the *L. sericata* pupae reared indoors were aged to the day thanks to the 3D plot obtained using the CHC analysis and PCA. Since the temperature effect was minimized as a constant temperature was used, it was easier to follow the changes between the cluster and estimate the age of puparia.

5.3.2 Discussion of Ageing Puparia

Studies of Diptera ageing have generally focused on larvae and empty puparia and rarely on adult flies. The ageing of the pupae is generally not preferred or involves complex and costly gene expression or imaging techniques. Estimating the age by extracting the CHC profiles of the pupae is a rare achievement due to the minimal quantity and diversity of CHCs in the chemical profiles of pupae compared to larvae and adult fly stages and the majority of the chemical profiles being composed of n-alkanes. While alkenes were hardly detectable at all, the number of branched methyl alkanes was also relatively small, which may cause problems when using CHC analysis.

In this study, the percentage concentrations of n-alkanes remained constant throughout the puparial period, even for C25:H, C27:H and C29:H n-alkanes. For this reason, it was unfortunately not possible to distinguish the quantitative amounts of CHCs by age as a basis. By eliminating n-alkanes, based on branched methyl alkanes and, if any, alkenes, an age-related separation could be obtained with PCA results.

Since the previous studies so far have been carried out under constant temperature, the primary purpose of this study was to reveal the effect of fluctuating temperature on the CHC profiles of the pupation. Therefore, adding to the work carried out outdoors, a control group was reared in an incubator at a constant temperature of $25\pm 1^{\circ}\text{C}$. Thus, the effect of temperature on the pupae chemical profiles was examined by comparing the control group pupae chemical profiles and those developing outdoors. As a result, the same results were reached regarding the percentage concentration of n-alkanes in pupae both reared outdoors and under constant temperature.

Chromatograms of pupae were nearly stable and similar throughout the entire puparial period. The percentage concentrations of n-alkanes other than C25:H, C27:H and C29:H remained almost constant. However, more CHC compounds in numbers and concentration were detected in the chemical profiles of outdoors reared pupae than pupae reared under constant temperature. C25:H, C27:H and C29:H n-alkanes, whose concentration varied depending on age, showed a regular increase or decrease in pupae reared at a constant temperature. OLA n-alkanes were found to be predominant for both species and in both experimental sets. A regular increase or decrease was not observed in

the pupae reared in the field and exposed to fluctuated temperature during the puparial period. This situation revealed a similar result that temperature had on the CHC profile of the larvae. Pupae formed more CHCs in order to resist weather conditions and temperature when exposed to fluctuating temperatures.

The study results showed that it is possible to estimate the age by applying PCA to the CHC data obtained from CHC profiles, even *C. vicina* and *L. sericata* pupae reared in the field or an incubator. Also, the temperature has a significant effect on the pupae stage as well as on the larval stage. Thus, along with the results, ageing to the day by CHC analysis is an important starting point for future studies.

5.4 Empty Puparia

Empty puparia, especially those of the Calliphoridae species, are the only entomological evidence encountered in advanced decay or dry remains where all other entomological evidence is no longer available [155]. With the end of the puparial period, adult flies emerge, and the empty puparia are simultaneously left at the crime scene and can stay even weeks or several years after death [45]. The interval between the adult fly eclosion and the collection of an empty puparia is called the post-eclosion interval (PEI) and is often overlooked [156]. The age of adult flies is estimated retrospectively using temperatures before the end of development. However, if it is not known when the development was completed and what temperatures will be used, and thus the thermal accumulation method cannot be used. Matuszewski and Mađra-Bielewicz [54] calculated the eclosion age of *P. terraenovae* empty puparia collected from the crime scene. However, because there was no evidence of when the adult flies first emerged, they used the average daily temperatures in the room. Due to the uncertain PEI and PIA, the PMI_{min} is likely to be overestimated in these cases. In this case, it is important to determine the age of empty puparia.

The CHC profile of pupae structure is very durable, so the CHC analysis can be used to predict PMI_{min} with much higher accuracy. Although there is little research about PEI estimation [43,78,157], primarily chemical analyses of pupae have been found to assist in determining PEI. Frere et al. [154] were able to distinguish empty puparia of

Hydrotaea aenescens as either recent (2012) or old (1997) by comparing the contents of their chemical profiles. Zhu et al. [158] determined the weather-related changes in the CHCs of *Chrysomya megacephala* empty puparia in the laboratory using GC-MS for up to 90 days. The results showed significant regular changes in the profile with age and mentioned factors that can affect the determination of the CHC composition of empty puparia, including the geographical populations in which the larvae and pupae live, sex, temperatures, humidity, food type, nutritional status and environmental characteristics such as wind, precipitation and sunlight.

Zhu et al. [157] conducted the same research with *Ch. megacephala* empty puparia in the field instead of in a laboratory environment. The results again showed that the relative concentration of branched alkanes and alkenes decreased significantly during the 90-day weathering process. In contrast to the work carried out in the laboratory, the changes in the CHC profiles of the empty puparia found in the field research were much more remarkable. In another research by Zhu et al. [159], to determine the change in CHC composition over age and to experience natural weathering, *Ch. rufifacies* empty puparia were placed on the field and found that the weathering of puparial CHCs was regular and highly predictable in the field. The percentage concentration of most CHCs has decreased significantly.

Moore et al. [43] examined the CHCs in the empty puparia chemical profiles of *C. vicina* and *L. sericata* species to estimate the age of empty puparia for nine months, and the results obtained under standardized laboratory conditions indicated that if insects stay with a corpse indoors for too long, they may use the same corpse for several life cycles, even if the corpse is in the drying phase. Paula et al. [155] determined PMI based on the chemical composition of empty puparia from three different generations of *Ch. megacephala* stored in the laboratory environment and showed that chemical profiles could be used to differentiate empty puparia collected from successive cycles.

In this section of the chapter, the CHC composition of empty puparia and environmental effects on ageing after the puparial stage is over and the adult flies emerge are investigated. For *C. vicina* empty puparia collected from outdoor-reared samples, the extraction period was limited to up to ten days. The experimental sets were stored in outdoor cages with adult flies without direct exposure to the natural weather conditions.

For *L. sericata* empty puparia collected from outdoor-reared samples, the extraction period was 90 days. They were buried in soil and were exposed to environmental conditions such as wind, rain and sunlight. For two species, the control groups were stored in a $25\pm 1^{\circ}\text{C}$ incubator in the laboratory to observe the temperature impact on CHC stability.

5.4.1 Results for Empty Puparia

5.4.1.1 *L. sericata* empty puparia

5.4.1.1.1 Fieldwork study

After adult flies hatched from *L. sericata* puparia were reared outdoors, the CHC profiles were extracted on Day 1. The remaining empty puparia were then shallow-buried in soil exposed to natural environmental conditions. After ten days, sufficient empty puparia were removed from the soil and extracted for Day 10. Then, at the end of 90 days in total, empty puparia were taken out from the soil again and were extracted for GC-MS analysis. Table 5.9 shows the identified CHCs extracted from the empty puparia from Day 1 to Day 90 and the total percentage and percentage standard deviation values of each compound. A total of 19 CHC compounds were identified in the GC chromatograms of empty puparia. The total CHC concentrations of CHC profiles were n-alkanes (42%) and branched methyl alkanes (58%). Extracts mainly contained saturated CHCs of C₂₅:H to C₃₃:H. Although the number of branched methyl alkanes predominated in the CHC profiles, the predominant compound was n-alkanes. Few alkenes were detected, with concentrations of less than 0.5%; thus, they were not included in the comparisons.

Table 5.9: CHC compounds extracted from *L. sericata* empty puparia stored outdoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 10	DAY 90
1	C25	4.71±0.87	6.16±1.73	3.48±2.27
2	C26	1.20±0.19	1.58±0.65	tr
3	C27	26.78±4.22	30.63±10.33	50.37±32.86
4	3-MeC27	4.05±0.89	3.73±2.04	3.54±2.89
5	C28	3.32±0.67	3.65±1.17	4.53±2.97
6	13+15-MeC28	0.71±0.18	tr	tr
7	3-MeC28	1.10±0.21	1.34±0.51	2.02±0.91
8	C29	33.17±5.67	35.11±8.22	28.36±15.03
9	13+15-MeC29	4.62±0.56	2.06±0.86	tr
10	7-MeC29	0.90±0.37	0.69±0.54	tr
11	4-MeC29	1.48±0.52	0.98±0.58	tr
12	3-MeC29	2.69±0.90	1.33±0.51	2.53±0.84
13	C30	1.40±0.49	1.32±0.51	tr
14	8-MeC30	0.68±0.34	tr	tr
15	3-MeC30	1.09±0.27	1.19±0.34	1.69±0.71
16	C31	9.06±1.84	9.31±2.66	3.48±1.45
17	13+15-MeC31	1.51±0.52	tr	tr
18	3-MeC31	0.79±0.28	tr	tr
19	C33	0.74±0.21	0.91±0.38	tr

tr = Trace amount detected (<0.5%)

When the chromatograms of the empty puparia were examined (Figure 5.21), it was observed that the number of n-alkanes decreased with age. Most of the branched alkanes detected on Day 1 dispersed after 90 days. Diminishing concentrations of CHCs either formed with other n-alkanes or were branched [158]. While changes during the first 10 days were minimal, after 90 days, the observed changes in chemical profiles were remarkable. The increase in the percentage concentration of C27:H n-alkanes at Day 90 was striking, while the percentage concentrations of C29:H and C31:H n-alkanes had decreased significantly.

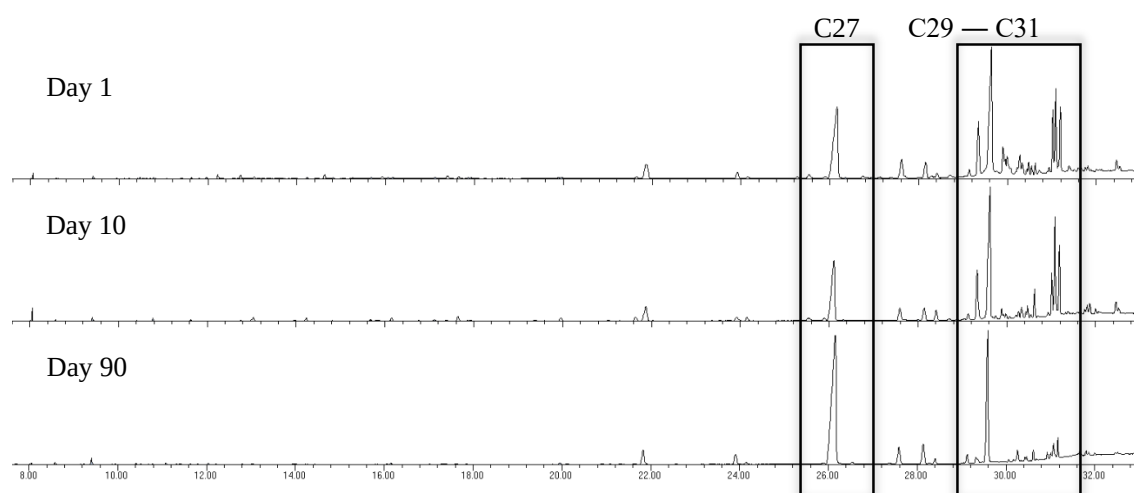


Figure 5.21: GC chromatograms of *L. sericata* empty puparia stored outdoors from Day 1, Day 10 and Day 90, respectively

The majority of branched methyl alkane concentrations in the empty puparia remaining in the soil for 90 days were dispersed or formed n-alkanes or (more commonly) other branched alkanes (Appendix 19). This explains why the percentage concentration of some CHCs decreases while the percentage concentration of others increases. The 3-MeC27 concentration decreased over age. The branched methyl alkanes 13+15-MeC28, 8-MeC30, 13+15-MeC31 and 3-MeC31 were detected only on the Day 1 chromatogram. The percentage concentrations of 13+15-MeC29, 7-MeC29 and 4-MeC29 branched methyl alkanes decreased from Day 1 to Day 10 and completely disappeared on Day 90. The percentage concentrations of 3-MeC28 and 3-MeC30 increased from Day 1 to Day 90.

When exploring the changes in the total concentrations of n-alkanes and branched methyl alkanes with age during the 90 days (Appendix 20), it was seen that there was a regular decrease in the CHC profiles of the empty puparia. When Day 1 and Day 90 extractions were compared, it was found that there was a 39% reduction in the percentage concentrations of n-alkanes and a 60% reduction in the percentage concentrations of branched methyl alkanes in the CHC profiles.

PCA calculations of *L. sericata* empty puparia stored outdoors buried in the soil were achieved by applying the CHC compounds given in Table 5.9. PCA was performed using six main components, explaining 99.8% of the variation in the data set. The three principal components were 95.7, 4.0 and 0.1%, respectively. PC2 and PC3 scores were used to form the plot given in Figure 5.22.

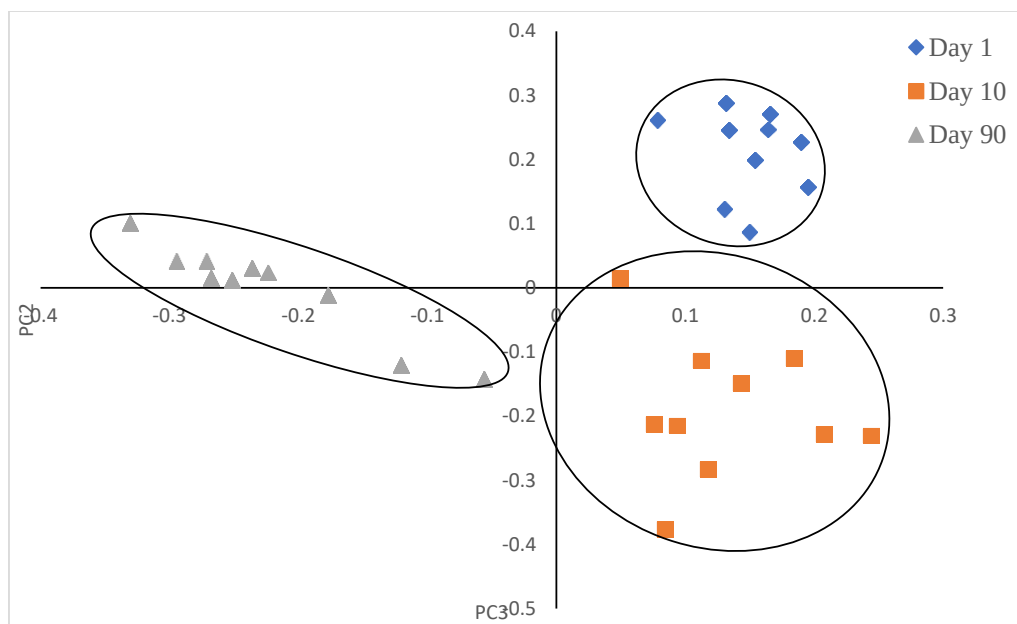


Figure 5.22: PCA plot showing PC3 against PC2 for *L. sericata* empty puparia stored outdoors, using n-alkanes and branched methyl alkanes with clustering days circled

The separation between clusters was evident in the 2-dimensional plane. It could follow the change in chemical profiles in a systematic direction. Clusters representing Day 1 empty puparia were seen at the top right on the plot, while Day 10 clusters were just below them. Day 90 clusters were in the left area of the plot. The percentage concentration of CHC compounds in the CHC profiles of empty puparia stored outdoors decreased over age. The change in CHC profiles of empty puparia within the first ten days and the significant change at the end of 90 days can be easily seen from the PCA plot. With the CHC analysis and PCA, *L. sericata* empty puparia have provided precise age estimation between clusters within the 90 days from Day 1 to Day 90. In addition, it also revealed the stability of CHC profiles of empty puparia stored in natural environmental conditions.

5.4.1.1.2 Control group at constant temperature ($25\pm1^{\circ}\text{C}$)

The procedure applied to empty puparia stored outdoors was also applied to empty puparia stored indoors to be able to compare. In total, *L. sericata* empty puparia stored at a constant $25\pm1^{\circ}\text{C}$ incubator yielded a profile of 17 identifiable CHC compounds from Day 1 to Day 90 (Table 5.10). The total CHC concentrations of CHC profiles were n-alkanes (47%) and branched methyl alkanes (53%), whose chain lengths range from C25:H to C33:H. Although the number of branched methyl alkanes predominated in the CHC profiles, the predominant group in concentration was n-alkanes. The alkenes detected were not considered because they were not in sufficient concentrations ($<0.5\%$).

Table 5.10: CHC compounds extracted from *L. sericata* empty puparia stored indoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 10	DAY 90
1	C25	25.21 $\pm$ 3.29	21.37 $\pm$ 4.64	18.05 $\pm$ 4.87
2	C26	0.78 $\pm$ 0.22	0.97 $\pm$ 0.27	1.08 $\pm$ 0.35
3	C27	16.97 $\pm$ 3.77	20.38 $\pm$ 5.45	22.98 $\pm$ 7.64
4	3-MeC27	2.94 $\pm$ 0.80	3.26 $\pm$ 0.93	3.75 $\pm$ 1.19
5	C28	2.55 $\pm$ 0.50	2.74 $\pm$ 0.63	2.97 $\pm$ 0.81
6	3-MeC28	1.41 $\pm$ 0.56	1.48 $\pm$ 0.40	1.41 $\pm$ 0.44
7	C29	24.18 $\pm$ 4.97	24.73 $\pm$ 5.34	25.53 $\pm$ 6.07
8	13+15-MeC29	5.73 $\pm$ 2.04	6.27 $\pm$ 1.77	5.82 $\pm$ 1.99
9	7-MeC29	1.12 $\pm$ 0.29	1.20 $\pm$ 0.37	1.17 $\pm$ 0.39
10	4-MeC29	1.00 $\pm$ 0.26	1.10 $\pm$ 0.24	1.00 $\pm$ 0.28
11	3-MeC29	0.84 $\pm$ 0.20	0.92 $\pm$ 0.26	1.04 $\pm$ 0.29
12	C30	1.36 $\pm$ 0.37	1.19 $\pm$ 0.29	1.19 $\pm$ 0.23
13	8-MeC30	0.60 $\pm$ 0.17	0.62 $\pm$ 0.17	0.58 $\pm$ 0.24
14	3-MeC30	1.49 $\pm$ 0.60	1.26 $\pm$ 0.33	1.19 $\pm$ 0.37
15	C31	10.02 $\pm$ 2.61	9.41 $\pm$ 1.65	9.33 $\pm$ 2.09
16	11+13-MeC31	2.68 $\pm$ 0.84	2.39 $\pm$ 0.69	2.24 $\pm$ 0.69
17	C33	1.13 $\pm$ 0.73	0.70 $\pm$ 0.18	0.66 $\pm$ 0.18

tr = Trace amount detected ($<0.5\%$)

Figure 5.23 shows the chromatograms of *L. sericata* empty puparia stored indoors for Days 1, 10 and 90, respectively. Peaks at retention times prior to C25:H were generally polar compounds. Although the chromatograms belonged to different ages, they were almost similar except for changes in the C25:H, C27:H, C29:H and C31:H peak areas. The C25:H concentration in the empty puparia stored indoors for 90 days decreased, while the C27:H concentration increased. In addition, while there was a 1% increase in C29:H with age, there was a 1% decrease in the percentage concentration of C31:H.

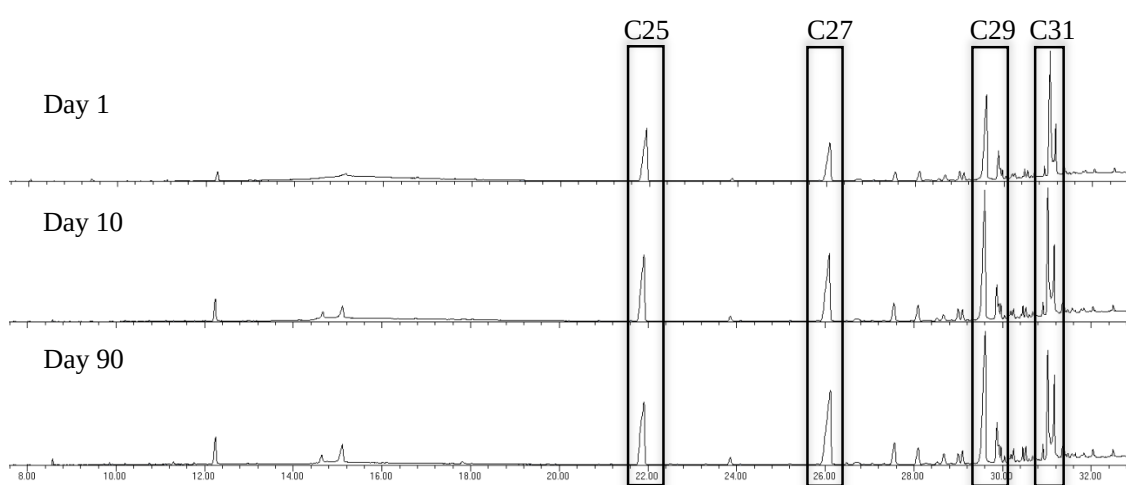


Figure 5.23: GC chromatograms of *L. sericata* empty puparia stored indoors from Days 1, 10 and 90

When the total concentration of CHCs in the profiles was examined according to the extraction days (Appendix 21), it was found that both the percentage concentrations of n-alkanes and branched methyl alkanes increased with age. In the empty puparia chemical profiles stored indoors, between Day 1 and Day 10, the percentage concentration of n-alkanes increased by 7%. The percentage concentration of branched methyl alkanes increased by 9%. At the end of 90 days, there was a total increase of 12% in the percentage concentration of n-alkanes and 13% in branched methyl alkanes.

The changes in the chemical profiles of the empty puparia stored outdoors buried in the ground and the empty puparia stored in a constant temperature incubator are shown in Figure 5.24. It was seen that significant decreases occurred in the percentage concentrations of total n-alkanes and branched methyl alkanes in empty puparia buried. On the other hand, the percentage concentrations of CHC compounds increased in empty puparia stored under constant temperature.

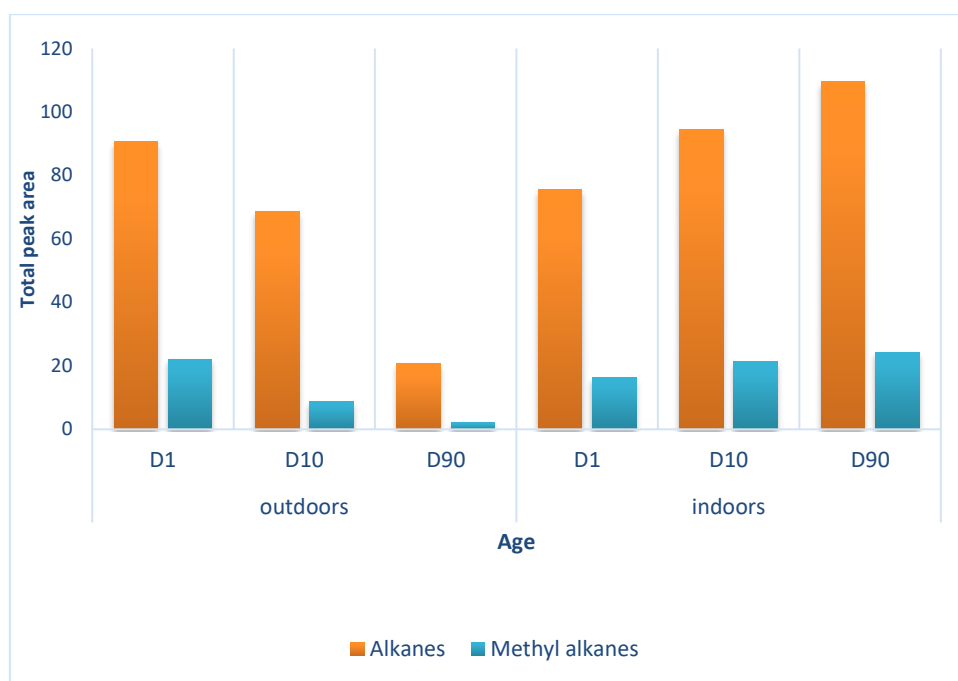


Figure 5.24: Plot showing the total concentration of n-alkanes and branched methyl alkanes found in the empty puparia of *L. sericata* stored outdoors and indoors

When the total n-alkane and branched methyl percentage concentrations obtained for both sets of experiments for 90 days were examined, although branched methyl alkanes predominated in number, the percentage concentration of n-alkanes was predominant. Since the CHC concentration in the chemical profiles of outdoors empty puparia decreased over age, the total concentration of both n-alkanes and branched methyl alkanes in the empty puparia stored indoors was more than in the empty puparia stored outdoors.

The percentage concentrations of C25:H, C27:H and C29:H n-alkanes detected in the chromatograms of the empty puparia stored outdoors and indoors were compared in Figure 5.25. The decrease in C25:H, C27:H and C29:H concentrations was remarkable in CHC compounds in the empty puparia stored outdoors. The percentage concentration of C25:H n-alkane decreased, while C27:H and C29:H concentrations increased in the empty puparia stored indoors.

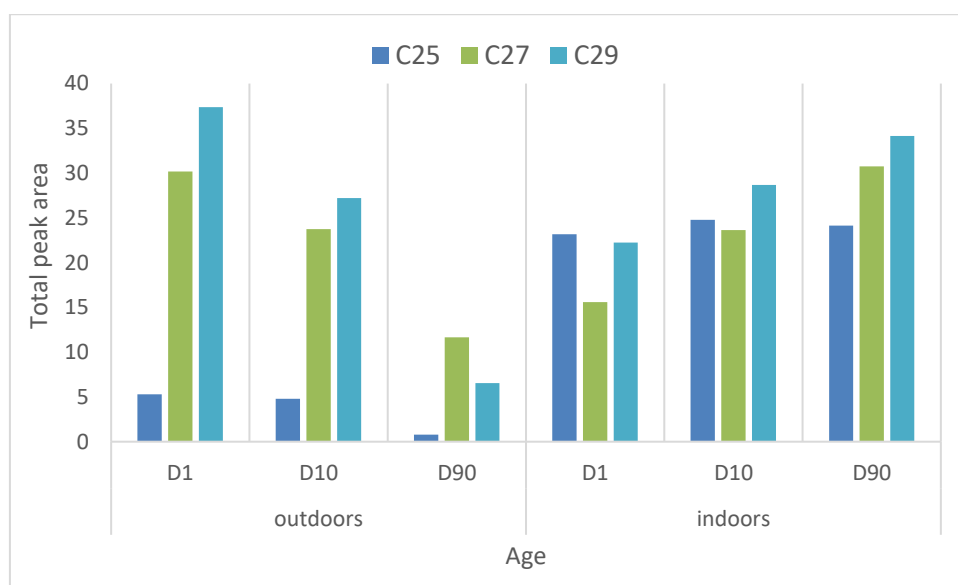


Figure 5.25: Plot showing the percentage concentration of C25:H, C27:H and C29:H n-alkanes present over the empty puparia of *L. sericata* stored outdoors and indoors

Since the changes in the chemical profiles were already minimal during this period spent in a reasonably protected environment at a constant temperature, a second PCA data set was created by eliminating the more stable n-alkanes from the original data set. PCA was performed using six main components, explaining 99.5% of the data set variation; the top three main components are 98.4, 0.7 and 0.3%, respectively. The PCA plot of *L. sericata* empty puparia stored at $25\pm 1^{\circ}\text{C}$ is given in Figure 5.26. The plot was formed using PC2 and PC3.

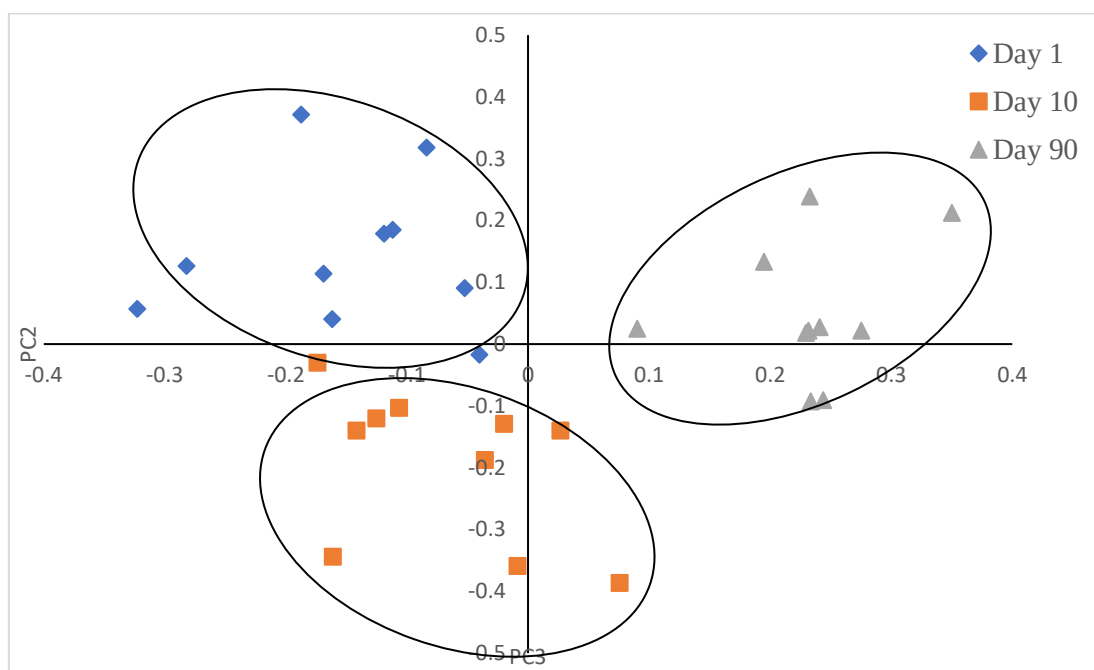


Figure 5.26: PCA plot showing PC3 against PC2 for *L. sericata* empty puparia stored indoors, using branched methyl alkanes only, with clustering days circled

Clusters representing Day 1 empty puparia are at the top-left of the plot, while Day 10 clusters are just below and Day 90 clusters are in the right area of the plot. The minimal change in CHC profiles of empty puparia within the first ten days and relatively greater change at the end of 90 days can be easily seen from the PCA plot. Although there was little difference in the CHC profiles of the empty puparia stored at $25\pm 1^{\circ}\text{C}$ constant temperature, age estimation could be made between Days 1, 10 and 90 due to CHC analysis and PCA. This method provides accurate age estimation and reveals the stability of CHC profiles of empty puparia stored indoors.

5.4.1.2 *C. vicina* empty puparia

5.4.1.2.1 Fieldwork study

After adult flies hatched from *C. vicina* pupae were reared outdoors, the remaining empty puparia were extracted for GC-MS analysis on Day 1 and then stored in cages placed in a field. After ten days, they were extracted and labelled as Day 10. In total, 34 CHC compounds with chain lengths ranging from C16:H to C33:H were detected. The total CHC concentrations of CHC profiles were n-alkanes (43%) and branched methyl alkanes (57%) (Table 5.11). The alkenes detected were not considered because they were not in sufficient concentrations (<0.5%). Although the number of branched methyl alkanes predominated in the CHC profiles, the predominant group in concentration was n-alkanes.

Table 5.11: CHC compounds extracted from *C. vicina* empty puparia stored outdoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 10
1	C16	0.77±0.46	tr
2	C18	1.27±0.82	0.58±0.08
3	4-MeC19	0.57±0.34	tr
4	3-MeC19	0.63±0.22	0.55±0.02
5	C20	1.24±0.69	0.86±0.07
6	C22	1.05±0.51	0.70±0.19
7	C23	0.81±0.51	0.73±0.17
8	C24	0.62±0.34	tr
9	C25	22.64±5.78	29.02±7.00
10	11+13-MeC25	0.67±0.33	tr
11	3-MeC25	1.60±0.91	1.31±0.45
12	C26	2.27±1.41	1.86±0.57
13	13+15-MeC26	1.11±0.51	1.15±0.20
14	3-MeC26	1.16±0.63	1.00±0.52
15	C27	25.11±13.33	23.22±6.22
16	13+15-MeC27	1.05±0.75	tr
17	11+13-MeC27	2.96±1.55	2.96±1.04
18	7-MeC27	1.17±0.55	1.20±0.38
19	4-MeC27	0.64±0.28	0.61±0.17
20	3-MeC27	6.81±3.15	6.64±2.00

Peak No.	Peak ID	DAY 1	DAY 10
21	5-MeC27	0.73±0.34	0.69±0.25
22	C28	2.12±0.89	1.94±0.56
23	13+15-MeC28	1.65±0.82	0.85±0.29
24	11+13-MeC28	0.54±0.28	0.56±0.19
25	4-MeC28	1.77±0.88	1.80±0.68
26	3-MeC28	tr	0.94±0.65
27	C29	9.54±2.99	10.14±2.23
28	13+15-MeC29	0.63±0.33	0.60±0.23
29	11+13-MeC29	2.48±1.05	2.51±0.88
30	7-MeC29	0.70±0.30	0.71±0.24
31	3-MeC29	0.82±0.39	0.88±0.21
32	8-MeC30	0.55±0.21	0.55±0.09
33	C31	1.97±0.65	2.23±0.43
34	C33	0.59±0.20	0.60±0.15

tr = Trace amount detected (<0.5%)

Figure 5.27 shows chromatograms of *C. vicina* empty puparia stored outdoors. There was no significant difference in other CHC concentrations when observing the chromatograms, except for C25:H, C27:H and C29:H n-alkanes. The chromatograms showed that C25:H and C29:H n-alkanes concentrations increased with age, while the percentage concentration of C27:H n-alkanes decreased.

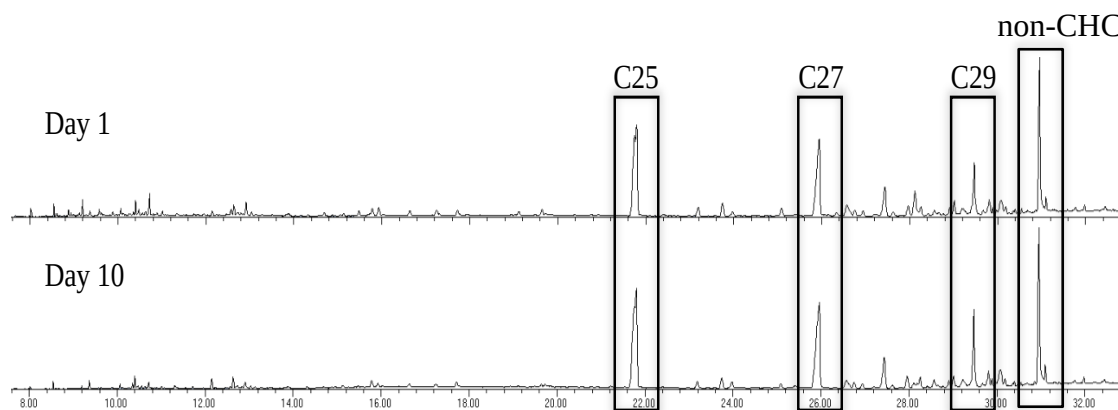


Figure 5.27: GC chromatograms of *C. vicina* empty puparia stored outdoors from Day 1 and Day 10

When the percentage concentration of n-alkanes and branched methyl alkanes were examined for ten days (Appendix 22), it was noticed that both the total n-alkane concentrations and the branched methyl alkane concentrations decreased by 5% and 10% (respectively).

It was difficult to separate the clusters on the PCA plot produced by applying the n-alkanes and branched methyl alkanes identified in the chromatograms with overlapping clusters. Because the changes in chemical profiles were already modest during a 10-day timeframe, a second PCA data set was created by removing the more stable n-alkanes from the first. The new PCA plot obtained using only the percentage concentrations of branched methyl alkanes compounds is given in Figure 5.28. It was performed using the first three main components containing 99.7, 0.1 and 0.1%, respectively and six main components explaining 99.9% of the data set variation.

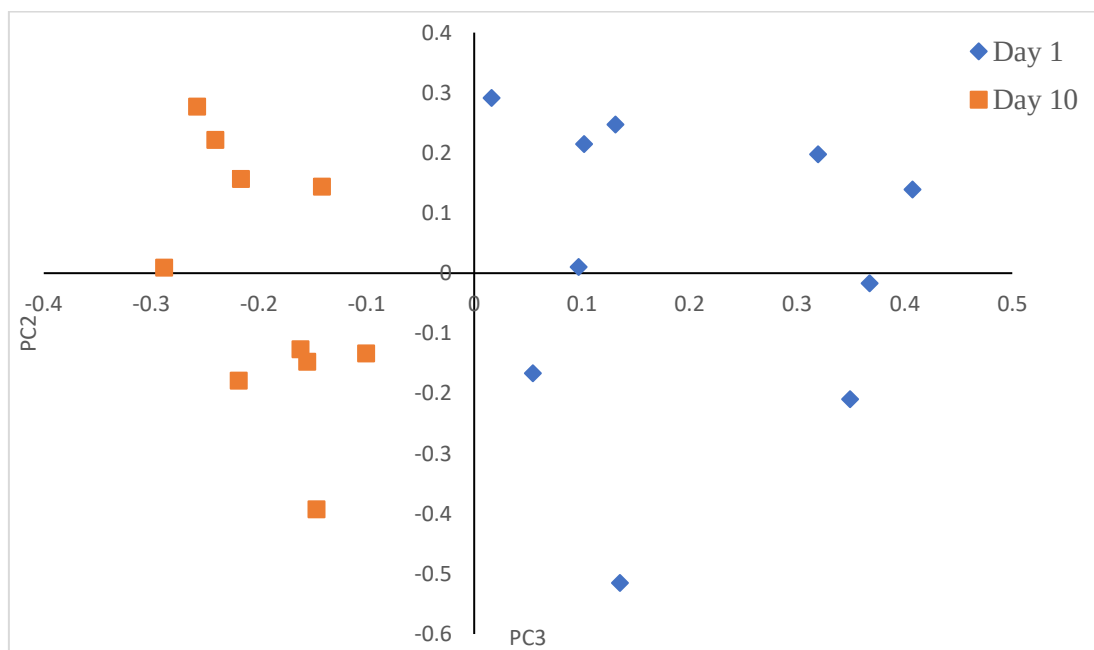


Figure 5.28: PCA plot showing PC3 against PC2 for *C. vicina* empty puparia stored outdoors, using only branched methyl alkanes

Clusters representing Day 1 empty puparia were seen in the right area, while Day 10 cluster was in the left area. With the aid of the CHC analysis and PCA, age estimation could be achieved between Days 1 and 10 based on the differences in the CHC profile.

The stability of CHC profiles of empty puparia stored outdoors was also revealed, although there was no significant difference in the chromatograms of *C. vicina* empty puparia. It should be noted that ten days is generally considered an insufficient period for ageing empty puparia, as they allow for a more extended period of age estimation (up to years).

5.4.1.2.2 Control group at constant temperature ($25\pm1^{\circ}\text{C}$)

In GC chromatograms of the extracted CHC profile of *C. vicina* empty puparia stored at a constant $25\pm1^{\circ}\text{C}$, 30 CHC compounds identified are given in Table 5.12. The total CHC concentrations of CHC profiles were n-alkanes (37%) and branched methyl alkanes (67%). In comparison, there were 2:3 ratios between the number of the n-alkanes and branched methyl alkanes detected in the chemical profiles. However, when their concentration was compared, the ratio between them was 3:2. In other words, the predominant group in numbers was the branched methyl alkanes, while the predominant group in concentration was the n-alkanes. The CHC chain lengths were from C20:H to C31:H but were mainly between C25:H and C31:H for Day 1 and Day 10, after eclosion.

Table 5.12: CHC compounds extracted from *C. vicina* empty puparia stored indoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 10
1	C20	tr	0.58±0.09
2	3-MeC20	1.41±1.10	tr
3	C25	4.45±0.85	4.37±1.39
4	3-MeC25	0.56±0.11	0.70±0.23
5	C26	3.68±1.32	2.37±0.80
6	13+15-MeC26	0.51±0.14	0.65±0.18
7	3-MeC26	0.91±0.35	1.24±0.52
8	C27	36.08±8.41	33.03±10.94
9	11+13-MeC27	4.13±0.99	4.92±1.75
10	7-MeC27	1.29±0.54	1.31±0.51
11	4-MeC27	0.98±0.19	1.14±0.40
12	3-MeC27	8.00±1.43	8.57±3.22
13	5-MeC27	1.11±0.24	1.29±0.47

Peak No.	Peak ID	DAY 1	DAY 10
14	C28	4.63±1.03	4.29±1.45
15	13+15-MeC28	1.20±0.46	1.73±0.99
16	11+13-MeC28	1.35±0.22	2.32±0.48
17	7-MeC28	0.57±0.15	0.77±0.22
18	5-MeC28	1.71±0.36	2.09±0.70
19	4-MeC28	0.50±0.12	tr
20	3-MeC28	2.21±0.61	2.61±0.81
21	C29	13.88±3.38	13.41±3.75
22	13+15-MeC29	0.71±0.15	0.77±0.30
23	11+13-MeC29	3.86±0.82	4.21±1.54
24	7-MeC29	1.16±0.25	1.25±0.48
25	5-MeC29	0.53±0.08	0.65±0.15
26	4-MeC29	0.82±0.67	0.56±0.11
27	3-MeC29	1.61±0.45	1.89±0.84
28	C30	0.51±0.12	0.50±0.10
29	8-MeC30	tr	0.80±0.21
30	C31	0.94±0.40	0.87±0.26

tr = Trace amount detected (<0.5%)

The GC chromatograms extracted from empty puparia are shown in Figure 5.29. The GC chromatograms were very similar; the only substantive difference was that C27:H and C29:H n-alkanes concentrations decreased from Day 1 to Day 10. With age, only a 3% reduction in C27:H concentration and a 1% reduction in C29:H concentration were observed for ten days.

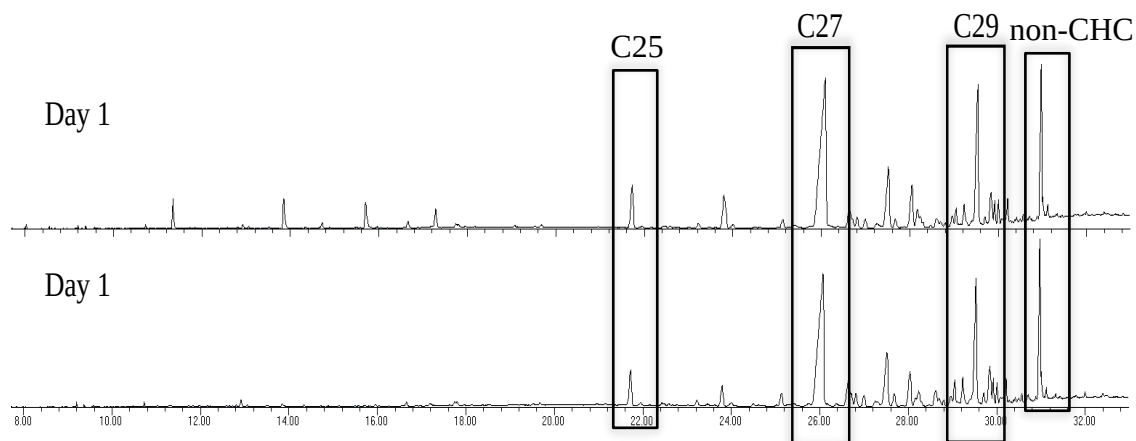


Figure 5.29: GC chromatograms of *C. vicina* empty puparia stored indoors from Day 1 and Day 10

Comparing the chromatograms, there was no noticeable change in the CHC profiles, only a decrease in the percentage concentrations of C27:H and C29:H n-alkanes. When the CHC concentrations of Day 1 and Day 10 were compared (Appendix 23), it was found that the total n-alkane concentration decreased by 4%. On the other hand, the percentage concentration of branched methyl alkanes increased by 6% in only ten days.

The effect of the fluctuating temperature on the stability of CHC profiles was investigated by comparing the CHC profiles of empty puparia left in the cage of adult flies in the field and stored in an incubator). Although branched methyl alkanes were found to be more numerous in both experimental sets, the predominant group in concentration was n-alkanes. The percentage concentration of n-alkanes in empty puparia stored under fluctuating temperatures was more remarkable than those stored under constant

temperature. Conversely, the branched methyl alkane concentration was more abundant in the CHC profiles of empty puparia stored under constant temperature.

C25:H, C27:H and C29:H n-alkanes were the ones whose concentration varied significantly in CHC compounds in both experimental groups. The changes in concentrations on Days 1 and 10 for *C. vicina* empty puparia stored outdoors and indoors are shown in Figure 5.30. The percentage concentration of C25:H was significantly higher in samples stored in the field and increased over the 10-day. The percentage concentration of C27:H was higher in samples stored under constant temperature, and it was the predominant n-alkane in this group. Its concentration decreased over age in both experimental sets. C29:H n-alkane was also found to be more abundant in empty puparia stored indoors than outdoors. Compared with the other two n-alkanes, the change in the percentage concentration of C29:H was relatively minor.

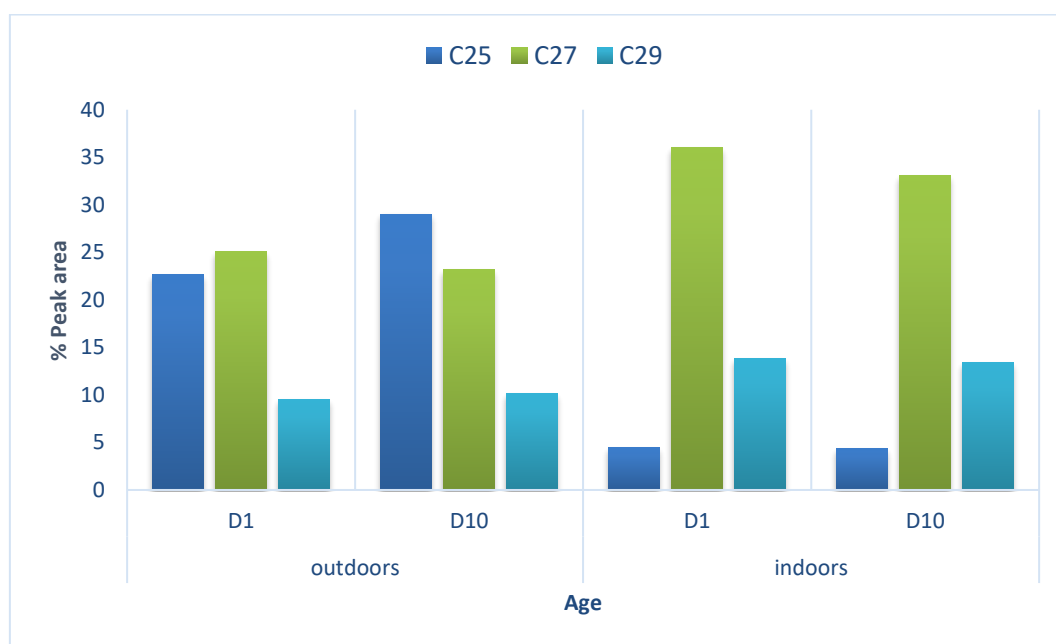


Figure 5.30: Plot showing the % concentration of C25:H, C27:H and C29:H n-alkanes present over the empty puparia of *C. vicina* stored outdoors and indoors

As the desired level of separation between age clusters could not be obtained in the PCA plot prepared using all CHCs in the chromatograms, only branched methyl alkanes were applied to generate the PCA plot. It was performed using six main components, explaining 98.9% of the data set variation; the top three main components are 97.6, 0.9 and 0.4%, respectively. The PCA plot of empty puparia of *C. vicina* stored at $25\pm1^{\circ}\text{C}$ is given in Figure 5.31, formed using PC2 and PC3.

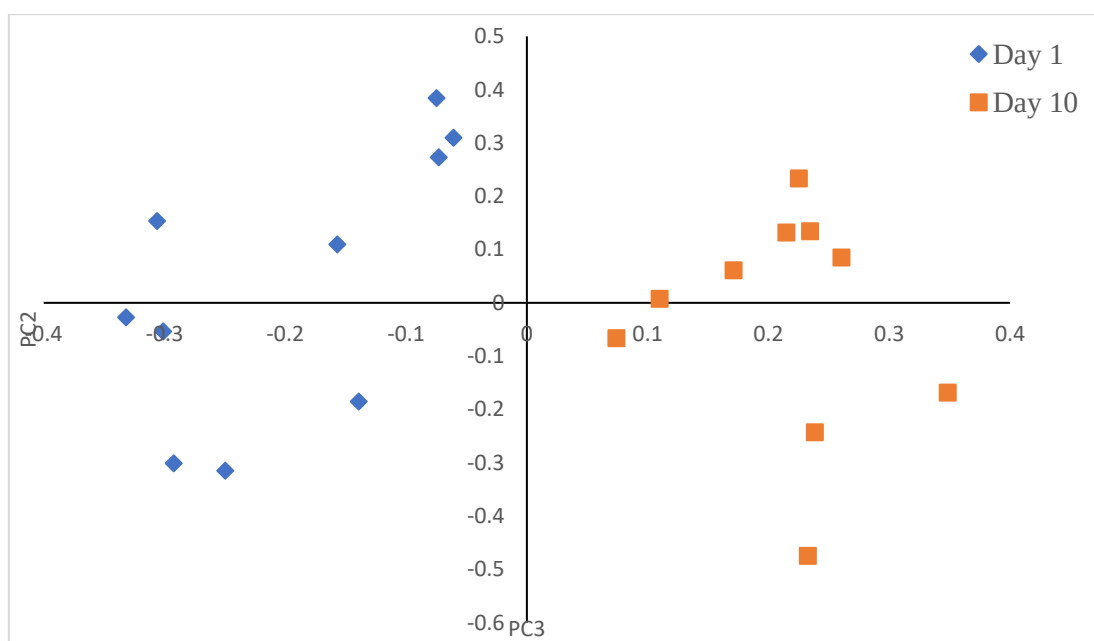


Figure 5.31: PCA plot showing PC3 against PC2 for *C. vicina* empty puparia stored indoors, using only branched methyl alkanes

Clusters representing Day 1 empty puparia are seen on the left, whereas Day 10 clusters were in the right area of the plot. Although there was little change in the CHCs in the empty puparia stored under constant temperature without exposure to any weathering or temperature change, the clusters separated even within a short period of ten days. Therefore, age estimation could be made between Days 1 and 10 with CHC analysis and PCA. This method provides accurate age estimation. In addition, it also reveals the stability of CHC profiles of empty puparia stored at a constant temperature.

5.4.2 Discussion of Ageing Empty Puparia

According to chromatograms of the empty puparia, it was complicated to discriminate the CHC profile changes. The changes in chemical profiles were much more minor, especially when stored under relatively protected conditions at a constant temperature. However, separation could be achieved with the CHC analysis and PCA to age the empty puparia even in a short period of ten days, according to the differences in the chemical profiles. The age of empty puparia usually includes months or years. However, due to the time constraint of the research, it was decided to limit it up to ten days for only *C. vicina* species and 90 days for *L. sericata* species.

Since, in the previous studies, the samples were reared at constant temperatures, the primary purpose of this study was to reveal the effect of fluctuating temperature on the stability of CHC profiles. Therefore, adding to the work carried out outdoors, a control group was reared in an incubator at a constant temperature of $25\pm 1^{\circ}\text{C}$ in the laboratory. Thus, the effect of temperature on the chemical profiles was examined by comparing the control group profiles and those developing outdoors.

The predominant group was generally n-alkanes, although the number of branched methyl alkanes constituted the majority in the empty puparia chemical profiles. When the change in the percentage concentrations of n-alkanes was examined by age, no significant change was observed in the percentage concentrations of n-alkanes other than n-alkanes with C25:H to C31:H carbon chain lengths. The percentage concentration of most shorter carbon chain CHCs decreased over age due to their lower molecular weight and higher volatility than CHCs with longer carbon chains.

The evaporation of shorter carbon chain CHC compounds from the chemical profiles causes an increase in the total percentage concentration of compounds with higher boiling points and longer carbon chains. Furthermore, branched methyl alkanes with longer chain lengths are cleaved from the branching point and transformed into n-alkanes [158]. This may be the reason for the increased concentration of C27:H n-alkane, which is an issue to be explored in future research.

The study explored *L. sericata* empty puparia exposed to natural weather conditions by being buried under the soil and stored in a protected environment under constant

temperature, revealing the environmental impact on weathering and ageing. In both conditions, besides age estimation with the difference in CHC profiles, the differences in environmental conditions were also clearly visible on the profiles. A significant decrease in the total concentrations and the number of CHC compounds were evident over age in *L. sericata* empty puparia exposed to outdoor weathering. Empty puparia stored in a relatively sheltered environment at a constant temperature, on the other hand, maintained CHC concentrations. The results obtained are compatible with the studies of Zhu et al. [157,158] in terms of the effect of indoor and outdoor spaces on the empty puparia profile. They reported that the chemical profiles obtained from the empty puparia changed significantly over the 90-day period and most of the compounds detected decreased with the weathering time. Although the empty puparia species used were different, the 90-day period in both studies is consistent in showing the effect of environmental factors on the CHC profile.

At the end of the research, it was found that the stability of CHCs and age are directly related to environmental conditions and temperature. With the differences in the CHC profiles of the empty puparia that occurred regularly over age, determining the age of the empty puparia and determining PMI_{min} is promising for future studies. This method can be applied for PMI_{min} estimates from 2-4 weeks to several months or even years. Generally, while ageing is based on months or even years in empty puparia, these results obtained in a short time of ten days and the classification of data with PCA support the increased importance of the method in forensic investigations.

5.5 Adult Fly Stage

In forensic entomology, the development of Diptera larvae and the time intervals of the larval stages are generally used for PMI_{min} determination, but these methods can only be used until the end of the metamorphosis and the emergence of adult flies. Estimating the age of adult flies can expand the predictable time window in which colonization occurs. In indoor crime scenes, where there are many living and dead adult flies or pupae or empty puparia, the ageing of adult flies can be an excellent solution for the estimation of PMI_{min}. While the empty puparia ageing offer scope for more extended periods of months

or years, the ageing of adult flies can provide a fine-tuned timing for the first days after the first wave of colonization hatches.

Adult fly samples found at a crime scene are usually needed for species identification. Techniques for accurately estimating the age of adult flies are still in their beginning stages or have not been adequately verified [160]. Studies on the morphological and physiological characteristics of adult flies required for age estimation are still ongoing. Some of the other approaches used to estimate the age of adult flies are fluorescence, gene expression, cuticle banding and volatile organic compound analyses [141]. Recent studies have shown that the CHC profile changes consistently based on adult fly age; thus, CHC analysis can be a powerful method to estimate the age of adult flies. Jackson and Bartelt [161] demonstrated that the CHCs of adult *Drosophila virilis* fruit adult fly varied with age and gender using TLC, GC, HPLC, GC-MS and ozonolysis-GC techniques. Tralalon et al. [144] examined changes in the CHC profiles of *C. vomitoria* adult flies 3-120 hours after hatching and found that the CHC compositions were different for both male and female adult flies and young (3 and 6 hours) and older (24 to 120 hours) flies.

Braga et al. [162] used CHC analysis with GC-MS to identify and age the female and male *Chrysomya putoria* adult flies from one to five days old. The results showed that it was possible to measure the relative concentration of the main peaks of male and female *Ch. putoria* for up to five days using cluster analysis. Bernhardt et al. [163] demonstrated that quantitative fluorescence analysis of pteridine in the heads of adult *C. vicina* adult flies could be used for age calculations, while Desena et al. [164] measured quantitative changes in whole-body CHCs of female *Aedes aegypti* mosquitoes that occur over age by using GC-FID. Later, Desena et al. [165] developed this method to use only the legs of female *A. aegypti*.

In addition, some CHCs, such as pentacosane (C₂₅:H) alone, can play an active role in estimating the age of adult flies. Bernhardt et al. [88] demonstrated that the percentage concentration of C₂₅:H in the legs of *L. sericata* and *C. vicina* adult flies increases with age and can thus be used for age prediction. With this study, it was shown that CHC analysis could be considered an economical method of using only the legs of the adult flies, allowing the rest of the adult fly body, such as the head or abdomen, to be used for further analysis. Moore [44] showed that age estimation could be made for five-day

periods (Days 1, 5 and 10) after the emergence of adult flies for *L. sericata* and *C. vicina*, but changes in the first five days were not known. Also, the stability of the CHC profiles of adult flies when reared outdoors is unknown.

This study aimed to use CHC profiles for age estimation of the adult fly stage of *L. sericata* and *C. vicina* reared outdoors. It has an unusual appearance when adult flies emerge from the empty puparia. The adult fly head has a protruding region called the ptilinum, inflated with blood, which allows them to exit the puparia, which subsequently retracts after a few hours, forming the ptilinal suture of the head. The adult fly wings are wrinkled, and the body colour is brownish/grey [40]. A few hours are given for the first-day extractions for this situation to pass and for the metamorphosis to complete. CHC profiles were obtained by extraction from Day 1 to Day 5 on a daily basis and then on Day 10.

5.5.1 Results for Adult Flies

5.5.1.1 *L. sericata* adult flies

5.5.1.1.1 Fieldwork study

Changes in CHC composition were studied for *L. sericata* adult flies between Days 1 and 10 after hatching. GC-MS chromatograms detected 33 CHCs, with the following total CHC profile concentrations: n-alkanes (28%), alkenes (12%) and branched methyl alkanes (60%) with chain lengths between C₂₄:H and C₃₂:H carbons. Branched methyl alkanes predominated both in number and in concentration in CHC profiles. The percentage concentrations of alkenes were almost equal to n-alkanes. Table 5.13 shows the present CHCs in the chromatograms and the total percentage and standard deviation values of each compound.

Table 5.13: CHC compounds extracted from *L. sericata* adult flies reared outdoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 10
1	C23	0.61±0.15	0.60±0.21	1.13±0.26	1.32±0.33	2.36±0.82	6.08±2.16
2	3-MeC24	tr	tr	tr	0.54±0.10	0.74±0.29	0.52±0.12
3	C25	2.76±0.66	2.43±0.83	2.91±0.60	3.28±0.73	4.00±0.96	9.38±3.48
4	11+13-MeC25	1.30±0.52	1.30±0.53	1.91±0.44	2.11±0.57	3.00±0.80	2.82±0.97
5	C26	0.72±0.19	0.83±0.31	0.77±0.15	0.71±0.20	0.68±0.17	0.99±0.30
6	8-MeC26	0.52±0.12	0.53±0.13	tr	tr	tr	0.58±0.17
7	3-MeC26	1.04±0.25	1.01±0.55	1.81±0.55	2.42±0.41	3.05±1.25	2.65±3.43
8	Heptacosene*	0.81±0.25	0.89±0.45	1.04±0.33	1.06±0.37	1.92±0.97	5.05±3.65
9	Heptacosene*	1.97±0.76	2.10±1.13	4.79±1.70	5.77±1.54	8.42±2.58	3.20±1.57
10	C27	8.65±1.87	9.08±3.33	7.95±1.76	8.18±2.48	7.78±1.79	18.11±6.16
11	13+15-MeC27	6.53±1.69	6.88±3.10	6.34±1.22	6.22±1.89	5.82±1.46	3.15±1.28
12	7-MeC27	0.63±0.19	0.69±0.25	0.50±0.10	0.51±0.15	tr	tr
13	3-MeC27	4.30±1.14	4.39±1.37	3.24±0.55	2.82±0.87	2.48±0.63	1.02±0.47
14	C28	1.18±0.34	1.06±0.41	0.85±0.19	0.83±0.30	0.71±0.19	0.93±0.30
15	13+15-MeC28	2.32±0.72	2.50±1.02	1.72±0.45	1.25±0.71	1.06±0.36	tr
16	3-MeC28	7.08±2.59	6.85±3.47	11.13±3.09	13.20±2.73	13.24±4.02	4.56±1.36
17	Nonacosene*	tr	tr	tr	tr	tr	12.59±12.21
18	Nonacosene*	6.35±1.44	5.71±2.81	7.91±1.57	7.29±2.18	7.42±2.01	2.67±2.31
19	Nonacosene*	3.77±1.36	2.35±1.02	4.09±0.97	4.32±1.00	4.46±0.92	0.85±0.52
20	C29	6.20±2.22	3.46±1.66	3.03±0.71	3.35±1.32	2.68±0.64	7.13±2.31

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 10
21	13+15-MeC29	tr	3.19±2.05	2.48±0.82	2.41±0.76	2.16±0.58	tr
22	11+13-MeC29	16.39±3.97	18.04±6.72	11.68±2.75	8.86±2.35	7.68±2.32	5.23±1.96
23	7-MeC29	1.64±0.73	tr	1.35±0.22	1.14±0.39	0.92±0.28	0.83±0.68
24	5-MeC29	3.82±1.42	3.83±1.47	2.47±0.58	2.12±0.70	1.66±0.54	0.54±0.26
25	3-MeC29	1.72±0.51	1.91±0.49	1.88±0.75	1.89±0.67	1.87±0.78	0.51±0.22
26	8-MeC30	1.52±0.45	1.61±0.86	1.44±0.36	1.66±0.39	1.01±0.33	tr
27	3-MeC30	5.25±1.22	4.53±1.80	5.86±1.18	7.00±1.71	4.91±1.97	3.42±1.49
28	C31	2.70±0.66	2.90±1.12	2.22±0.80	1.81±0.65	1.88±0.67	1.96±0.46
29	11+13-MeC31	2.72±2.19	4.02±1.46	2.69±0.63	2.27±0.63	2.00±0.52	2.08±0.55
30	3-MeC31	3.27±0.60	3.35±0.94	2.30±0.47	1.98±0.59	1.70±0.32	1.30±0.35
31	C32	0.58±0.30	tr	0.55±0.08	0.50±0.15	0.65±0.18	tr
32	5-MeC32	0.51±0.09	0.53±0.14	tr	tr	tr	tr
33	3-MeC32	tr	0.64±0.10	0.52±0.11	tr	tr	tr

tr = Trace amount detected (<0.5%)

* Double bond positions were not determined

The first five days and Day 10 GC chromatograms of adult fly extraction are given in Figure 5.32. In the CHC profiles of adult flies, long-chain CHC compounds were detected intensively. While the chromatograms obtained on Days 1 and 2 of the adult fly stages were almost identical, some differentiation started on Day 3. Although Days 3, 4 and 5 had the same compound peaks in terms of CHC, the compound concentrations changed with age. On the other hand, fewer compounds were detected in the Day 10 chromatogram, but the peak areas (concentration) of compounds were larger.

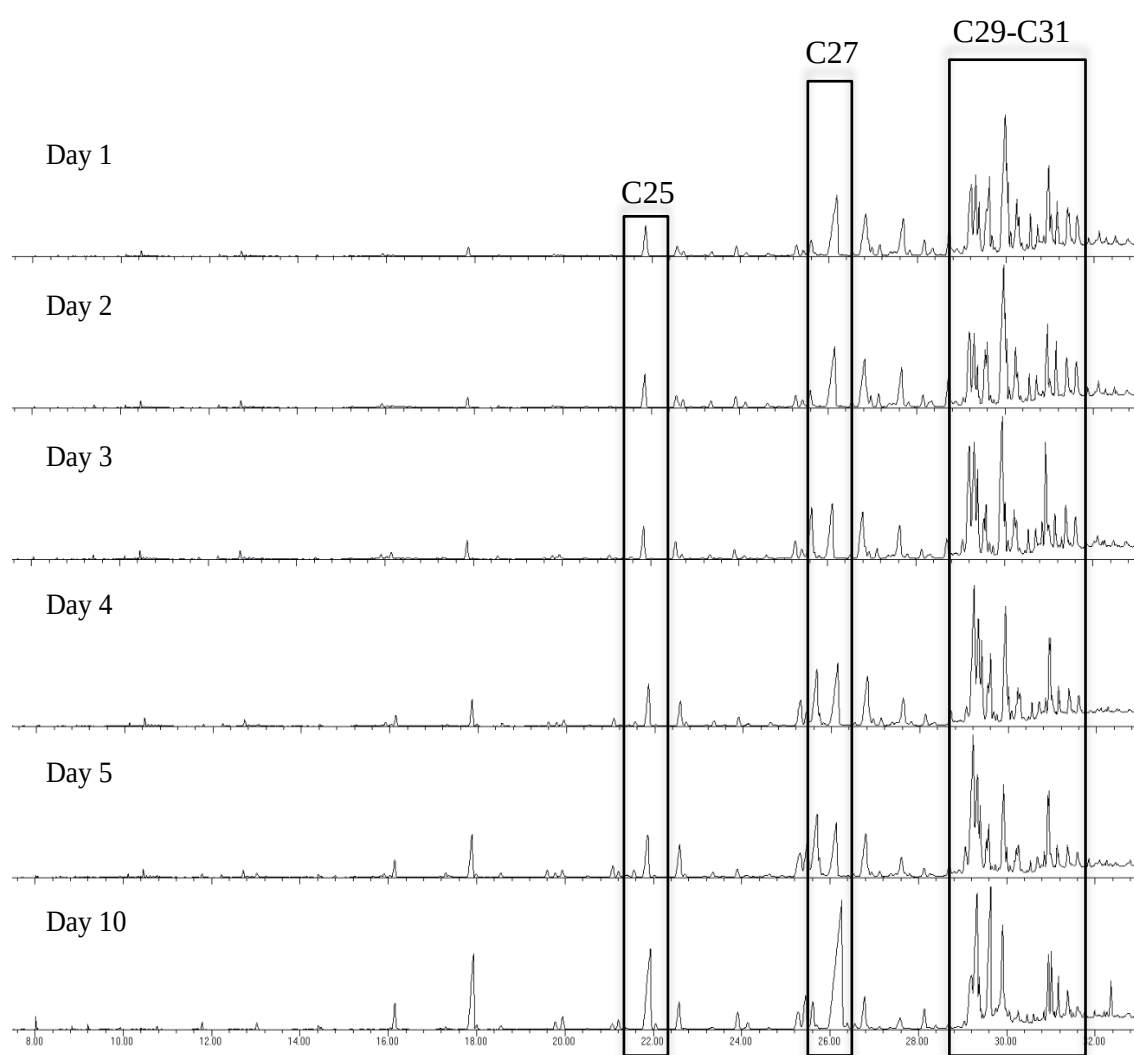


Figure 5.32: GC chromatograms of *L. sericata* outdoor adult flies from Day 1 to Day 10

The change over age of the percentage concentrations of n-alkanes detected in GC chromatography of the CHC profiles over ten days is given in Appendix 24. Accordingly, it was determined that OLA compounds were more concentrated in the chemical profiles than ELA compounds and their concentrations significantly changed with age. From Day 1 to Day 10, the percentage concentration of short-chain length n-alkanes increased, whereas the percentage concentration of n-alkanes with longer chain length decreased. Day 10 was the day when OLA compounds had the most intense concentration compared to previous days.

Considering the distribution of the total concentrations of the detected CHC compounds by age (Figure 5.33), it was obtained that branched methyl alkanes were abundant in the chemical profiles, both in number and concentration. From Day 1 to Day 5, approximately 60% of the chemical profiles consisted of branched methyl alkanes, while the rest consisted of n-alkanes and alkenes. On Day 10, this situation changed, and n-alkanes became more predominant in the chemical profile. In contrast, the branched methyl alkanes concentration was reduced. Thus, Day 10 was distinguished from previous days by the higher concentration of n-alkanes.

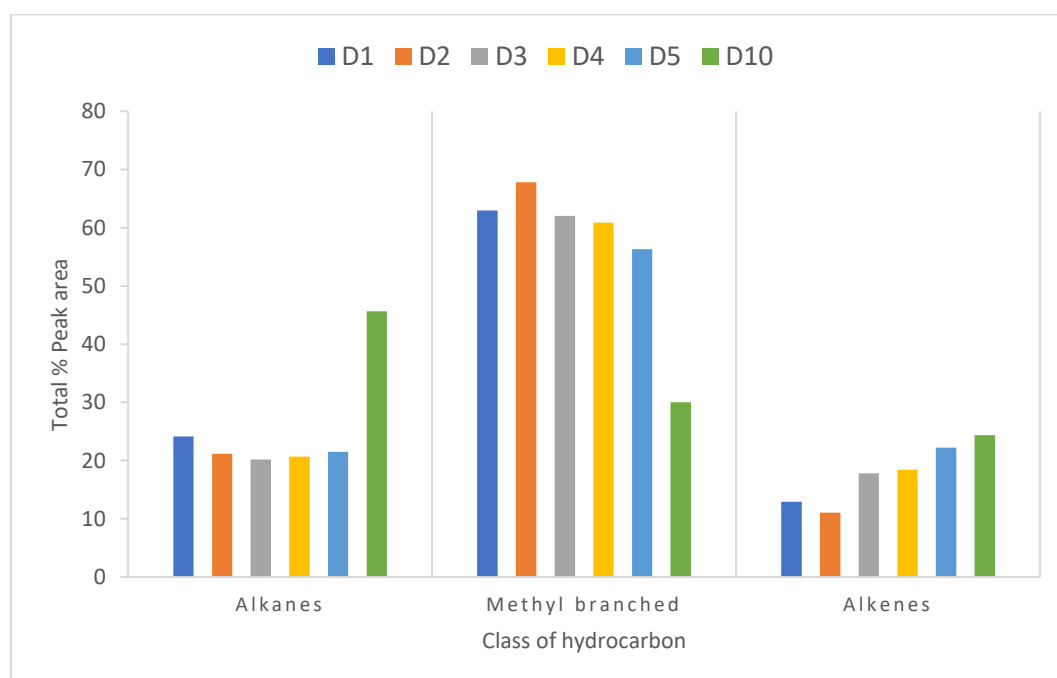


Figure 5.33: Plot showing the total percentage peak area of n-alkanes, branched methyl alkanes and alkenes of *L. sericata* outdoor adult flies from Day 1 to Day 10

The longer carbon chain branched methyl alkanes were the most abundant in chemical profiles and changed with age. The percentage concentrations of 3-MeC27, 5-MeC29, 3-MeC31 and 11+13-MeC29 decreased in a particular order over age. While the 3-MeC28 concentration was at the same level on Days 1 and 2, it increased on Days 3, 4 and 5 and decreased significantly on Day 10. If the adult fly age was estimated by looking only at these variables obtained from chromatograms, the above CHC compounds could be a clue for this aim. However, using the chromatograms was both time-consuming and somewhat difficult, given that they looked almost similar. At this point, PCA was evidently an easier and faster method for ageing clusters based on CHC profiles.

When the n-alkane, alkene and branched methyl alkane compounds obtained from the chromatograms and given in Table 5.13 were applied to PCA, the separation between the age clusters was obtained (Figure 5.34). PC2, PC3 and PC5 were the scores providing the best separation between clusters. PCA was performed using six main components explaining 97.1% of the data set variation, with the first three principal components being 82.4, 9.6 and 5.0%, respectively.

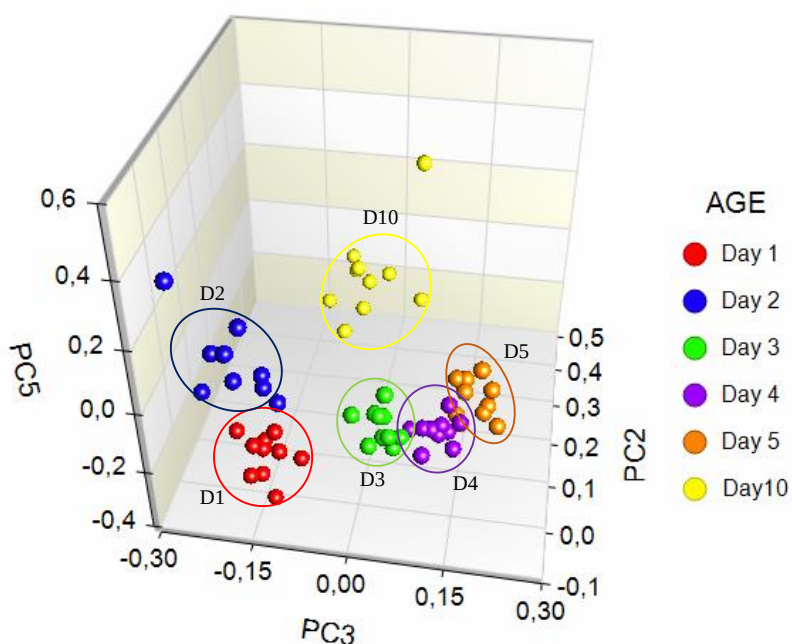


Figure 5.34: PCA 3D plot showing PC5 vs PC3 vs PC2 for *L. sericata* outdoor adult flies, using n-alkanes, alkenes and branched methyl alkanes, with clustering days circled

With PCA, the chemical changes occurring in the first five days can be differentiated even if they are barely visible in their chromatograms. Despite how chemically similar they were, they provided sufficient age estimation data. The separation between the clusters was based on the changes in the percentage concentration of CHC compounds. The clusters of Days 1 and 2 and Days 3, 4 and 5, which had very similar chemical profiles in their chromatograms, revealed the change in their chemical profiles as well as estimated age. Day 10 could be distinguished entirely from previous days. Thus, age estimates were made for Days 1, 2, 3, 4, 5 and 10 of *L. sericata* adult flies reared in a cage outdoors. It is also clearly noticeable that there were distinct chemical changes associated with age. With the CHC analysis and PCA, *L. sericata* adult flies were ageing to the day within ten days, despite the fluctuating temperature.

5.5.1.1.2 Control group at constant temperature ($25\pm 1^{\circ}\text{C}$)

A control group was reared in an incubator at a constant temperature of $25\pm 1^{\circ}\text{C}$ to observe the temperature effect on the CHC profiles of *L. sericata* adult flies and to age them. In total, *L. sericata* yielded a profile of 27 CHC compound peaks identified from Day 1 to Day 10 (Table 5.14). The total CHC concentrations of CHC profiles were n-alkanes (28%), alkenes (8%) and branched methyl alkanes (64%) for adult flies. The chain lengths ranged from C23:H to C32:H. Branched methyl alkanes predominated both in number and in concentration in CHC profiles.

Table 5.14: CHC compounds extracted from the *L. sericata* adult flies reared indoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 10
1	C23	tr	1.85±0.35	3.17±1.13	4.76±1.13	5.71±1.27	8.44±3.10
2	C25	1.82±0.46	4.11±0.73	6.03±1.90	7.23±2.15	7.65±1.61	11.09±3.59
3	11+13-MeC25	0.70±0.30	2.21±0.24	3.17±0.86	2.68±0.63	2.44±0.47	2.48±0.60
4	C26	0.86±0.16	0.96±0.15	1.16±0.40	0.96±0.25	0.86±0.15	1.09±0.28
5	3-MeC26	0.64±0.24	2.05±0.38	2.57±0.66	1.91±0.89	1.33±0.27	tr
6	Heptacosene*	0.78±0.67	7.89±0.86	13.67±4.65	18.58±6.13	14.88±4.32	6.32±1.63
7	C27	10.30±1.80	10.37±1.41	13.84±4.06	15.32±5.11	16.14±3.22	20.94±4.88
8	13+15-MeC27	4.53±1.58	8.07±1.59	8.63±3.01	5.62±1.41	4.43±1.07	2.51±0.57
9	5-MeC27	0.54±0.14	0.65±0.13	tr	tr	tr	tr
10	3-MeC27	4.94±0.39	4.09±0.76	3.40±1.64	2.16±0.77	1.54±0.35	0.85±0.32
11	C28	1.59±0.23	1.10±0.12	1.24±0.59	1.27±0.96	0.87±0.17	0.87±0.15
12	13+15-MeC28	2.64±0.43	2.40±0.70	1.98±0.98	0.88±0.42	0.51±0.13	tr
13	3-MeC28	6.15±2.59	tr	tr	tr	tr	tr
14	Nonacosene*	tr	tr	tr	tr	tr	6.67±1.48
15	C29	9.27±1.50	4.61±1.79	5.23±2.30	7.62±2.63	8.46±1.30	8.31±1.15
16	13+15-MeC29	21.88±2.55	17.45±4.05	14.55±4.68	13.69±4.18	11.72±3.25	6.88±0.65
17	9-MeC29	3.43±0.51	2.63±0.67	1.88±1.05	1.18±0.35	tr	tr
18	3-MeC29	1.70±0.18	1.49±0.29	1.56±0.75	1.47±0.59	1.13±0.37	1.57±0.72
19	C30	0.50±0.08	0.53±0.06	0.57±0.39	0.57±0.38	0.52±0.09	1.14±0.42
20	8-MeC31	2.79±0.43	2.42±0.51	2.55±1.28	1.58±0.55	0.89±0.26	0.67±0.33

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 10
21	3-MeC31	5.80±1.76	10.76±3.08	tr	tr	10.04±3.16	4.26±1.40
22	Hentriacontene*	tr	tr	1.04±0.46	1.09±0.25	0.92±0.28	4.62±0.59
23	C31	4.97±0.75	3.52±0.72	2.88±1.04	1.98±0.42	1.95±0.38	2.26±0.32
24	11+13-MeC31	6.69±0.72	3.94±0.90	3.70±1.61	3.59±0.99	3.76±0.88	3.77±0.86
25	3-MeC31	3.79±0.54	2.65±0.56	2.56±0.92	2.31±0.94	2.39±0.74	3.12±0.46
26	C32	0.61±0.14	0.74±0.10	0.80±0.29	0.77±0.28	0.56±0.17	0.51±0.21
27	3-MeC32	0.59±0.12	0.55±0.12	0.68±0.17	0.64±0.15	0.65±0.10	0.56±0.13

tr = Trace amount detected (<0.5%)

* Double bond positions were not determined

GC chromatograms of *L. sericata* in/outdoor adult flies are given in Figure 5.35, belonging to Day 1 and Day 10. Comparing the chromatograms, it can be seen that they had similar chemical profiles, especially for Day 1, except for some minor concentration differences. Day 10 chromatograms likewise showed a similar similarity. This proves how difficult it is to determine temperature impacts on CHC profiles using only chromatogram comparison.

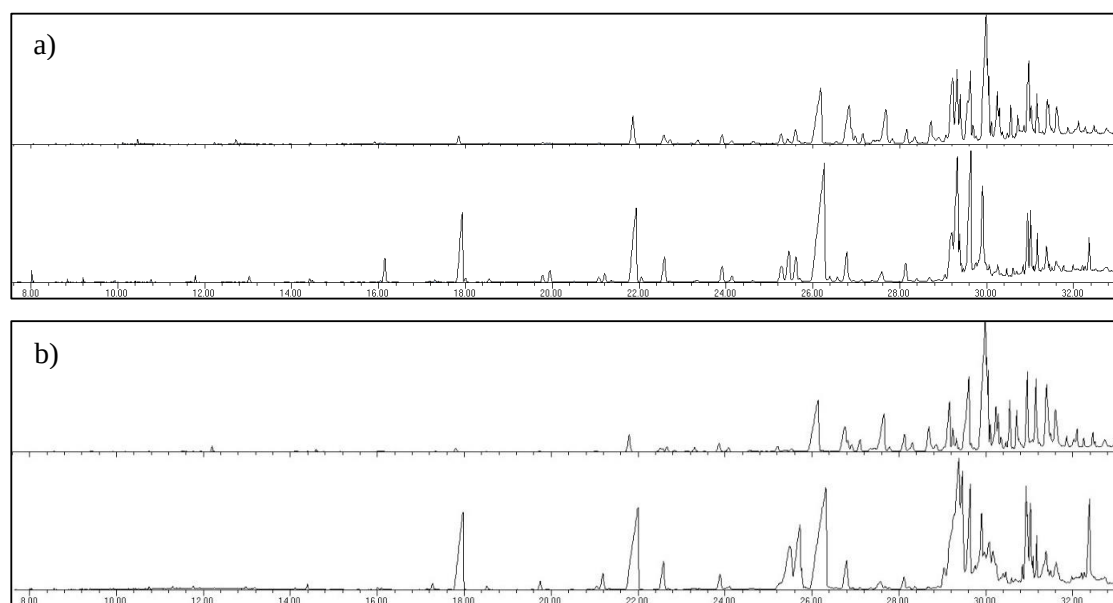


Figure 5.35: GC chromatograms of *L. sericata* adult flies reared in the UK for Day 1 and Day 10, respectively, reared a) outdoors b) indoors

When the percentage concentrations of n-alkanes detected in the chromatograms were examined (Appendix 25), it was seen that OLA compounds dominated the CHC profile, as observed in adult flies reared outdoors. However, unlike the OLA concentration of those, the OLA concentrations of indoor adult flies showed a steadily increasing change with age. Day 10 samples had the highest n-alkane concentration intensity. The variation of selected branched methyl alkanes was abundant in the chemical profiles and changed over age. Except for the increase of 13+15-MeC27 from Day 1 to Day 2, branched methyl alkane concentrations tended to decrease over the ten days.

When examining the change in percentage concentrations of CHCs during the 10-day extraction of *L. sericata* indoor adult flies (Figure 5.36), it was found that the percentage concentrations of n-alkanes and alkenes increased from Day 1 to Day 10, and the percentage concentration of branched methyl alkanes decreased day by day. Accordingly, Day 1 had the highest branched methyl alkane concentration, while Day 10 had the highest concentrations of n-alkanes and alkenes.

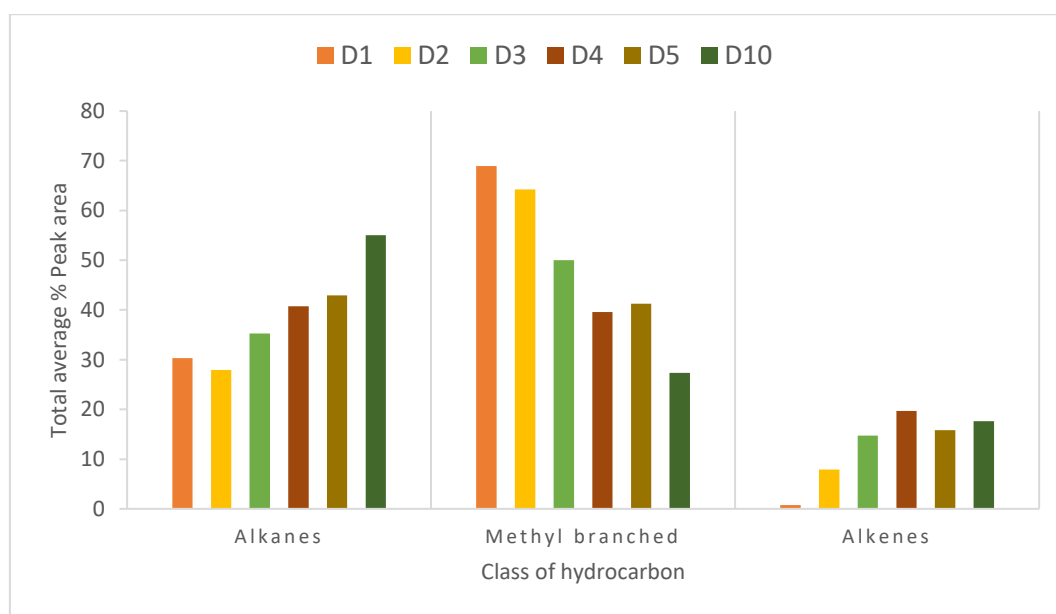


Figure 5.36: Plot showing the total percentage peak area of n-alkanes, alkenes and branched methyl alkanes of *L. sericata* indoor adult flies from Day 1 to Day 10

Although similarities of the chromatograms suggested that the CHC profiles of adult flies were affected by the temperature at a minimum level, differences emerged when the CHC profiles of indoor adult flies were detailed and examined. Interestingly, when the indoors and outdoors samples were compared in terms of the concentration of n-alkanes, it was seen that the indoor adult flies had a higher concentration of n-alkanes. In the larval and puparial samples of *L. sericata* species examined in previous sections, the total concentration of n-alkanes in samples reared outdoors under fluctuating temperatures was consistently higher than in samples reared at a constant temperature. When the branched methyl alkanes concentrations were observed, it was found that samples exposed to fluctuating temperatures had a higher concentration than the other sample set. Similarly,

samples reared under fluctuating temperatures had higher concentrations of alkene concentrations.

The percentage concentrations of CHC compounds changed with age between Days 1 and 10 is shown in Appendix 26. According to the plot, in the CHC profiles of adult flies reared under fluctuating temperature, both n-alkane and alkene concentrations increased by 28%. The percentage concentration of branched methyl alkanes decreased by 38%. For the CHC profiles of adult flies reared under constant temperature, while the n-alkane concentration increased by 28%, alkene concentration increased by 91%, and the percentage concentration of branched methyl alkanes decreased by 44%. As the adult flies got older in both experiment sets, the percentage concentration of n-alkanes and alkenes in their chemical profiles increased, whereas the branched methyl alkanes decreased.

In addition to examining the effect of indoor and outdoor rearing on the CHC profiles of adult flies, age estimates of adult flies reared at constant temperature were also explored. The PCA was carried out for *L. sericata* adult flies reared at $25 \pm 1^\circ\text{C}$, using six principal components, describing 96.2% of the variation within the dataset. The first three principal components comprise 83.7, 8.3 and 4.2%, respectively. To prepare the PCA data, n-alkanes, alkenes and branched methyl alkanes detected in the profiles were applied. In Figure 5.37, PC2, PC3 and PC5 were used to plot the relevant scores.

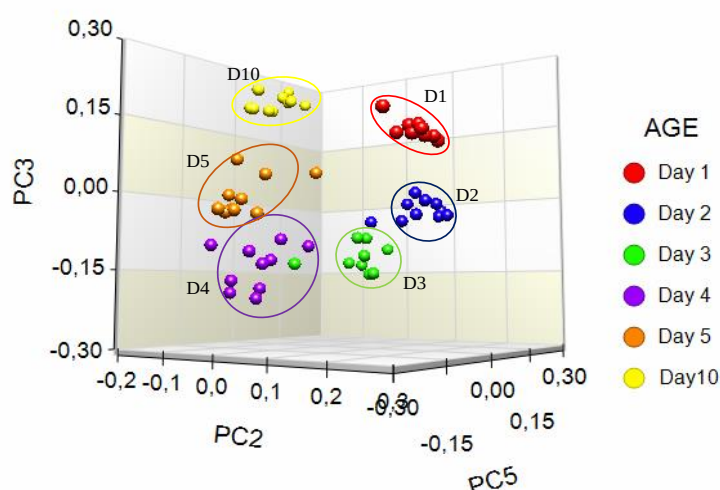


Figure 5.37: PCA 3D plot showing PC3 vs PC2 vs PC5 for *L. sericata* indoor adult flies, using n-alkanes, alkenes and branched methyl alkanes, with clustering days circled

The chemical change between the age clusters can be followed on the PCA plot, and the separation can be seen to estimate the age. Chemical changes between clusters could be traced in a systematic direction that moves clockwise. These results demonstrate the ability of the method for age estimation, especially in the first five days of ageing, based on changes and differences in CHC profiles with the aid of PCA.

5.5.1.2 *C. vicina* adult flies

5.5.1.2.1 Fieldwork study

Adult flies emerging from *C. vicina* pupae reared outdoors could not be extracted on the first and second days after eclosion. However, extractions were obtained from adult flies on Days 3, 4, 5 and 10. A total of 43 CHC compounds were detected in the GC chromatogram obtained from these extractions, as given in Table 5.15. It shows the CHCs present in the chromatograms, each total compound percentage and the percentage standard deviation values. The total CHC concentrations of CHC profiles were n-alkanes (24%), alkenes (6%) and branched methyl alkanes (70%). The predominant group in concentration and number was branched methyl alkanes in the chemical profiles. The chain lengths of the CHCs ranged from C22:H to C31:H. Although only one pentacosene was identified in the chemical profiles, it was considered as three different alkene compounds due to the double bond location change; the location of the double bond was not specifically given.

Table 5.15: CHC compounds extracted from *C. vicina* adult flies reared outdoors, total percentage of each compound present and percentage standard deviation for Days 3, 4, 5 and 10

Peak No.	Peak ID	DAY 3	DAY 4	DAY 5	DAY 10
1	C22	1.21±0.24	tr	tr	tr
2	3-MeC22	tr	tr	3.23±1.40	4.93±1.62
3	C23	0.94±0.32	2.56±2.07	3.83±1.59	7.65±2.35
4	9-MeC23	0.55±0.22	1.11±1.07	1.30±0.49	2.33±1.06
5	7-MeC23	tr	0.50±0.34	0.61±0.20	0.88±0.42
6	3-MeC23	1.00±0.25	0.94±0.32	1.03±0.24	0.99±0.34
7	C24	1.04±0.21	0.52±0.16	0.53±0.16	0.52±0.18

Peak No.	Peak ID	DAY 3	DAY 4	DAY 5	DAY 10
8	3-MeC24	2.52±0.94	10.61±4.25	tr	tr
9	Pentacosene*	0.86±0.38	tr	tr	tr
10	Pentacosene*	tr	1.41±0.81	19.42±3.14	29.96±13.42
11	Pentacosene*	tr	0.93±0.71	1.66±0.46	tr
12	C25	4.08±1.05	8.27±1.51	9.25±2.18	11.44±3.95
13	9-MeC25	5.38±1.61	5.73±1.26	4.97±1.82	4.71±3.65
14	7-MeC25	1.12±0.30	0.81±0.12	0.70±0.10	1.05±0.74
15	3-MeC25	2.91±0.70	2.34±0.33	1.98±0.36	1.25±0.48
16	C26	1.53±0.32	1.03±0.15	0.86±0.20	0.56±0.20
17	13+15-MeC26	1.45±0.38	0.98±0.12	0.70±0.11	tr
18	11+13-MeC26	1.26±0.33	0.97±0.29	0.53±0.09	tr
19	8-MeC26	0.68±0.23	1.11±0.64	tr	11.20±3.41
20	4-MeC26	7.91±2.38	12.98±3.28	13.05±3.62	tr
21	C27	9.99±2.18	10.14±2.04	8.26±1.95	7.20±2.92
22	11+13-MeC27	10.71±2.56	7.25±1.11	4.96±1.02	1.77±0.92
23	7-MeC27	2.34±0.55	1.59±0.23	1.04±0.21	tr
24	6-MeC27	0.79±0.21	0.59±0.10	0.51±0.11	tr
25	5-MeC27	1.40±0.37	0.80±0.09	0.52±0.09	tr
26	4-MeC27	0.62±0.15	0.50±0.08	tr	tr
27	3-MeC27	4.84±1.26	3.00±0.47	2.22±0.45	0.95±0.40
28	C28	1.31±0.29	0.83±0.16	0.66±0.14	tr
29	13+15-MeC28	tr	0.71±0.07	0.51±0.07	tr
30	11+13-MeC28	1.77±0.41	tr	tr	tr
31	8-MeC28	1.50±0.37	tr	tr	tr
32	4-MeC28	6.03±1.63	5.71±1.53	4.65±1.32	2.60±1.15
33	3-MeC28	1.60±0.41	1.68±1.20	0.89±0.42	tr
34	C29	4.01±1.39	2.74±0.98	2.43±0.45	1.64±0.64
35	11+13-MeC29	4.49±1.15	2.62±0.34	1.81±0.29	0.93±0.40
36	7-MeC29	1.01±0.26	0.64±0.09	0.50±0.08	tr
37	4-MeC29	tr	1.32±0.22	1.01±0.16	0.51±0.14
38	3-MeC29	3.90±1.91	0.77±0.23	0.63±0.09	tr
39	8-MeC30	1.03±0.25	0.64±0.18	0.52±0.09	tr
40	4-MeC30	0.89±0.26	0.85±0.19	0.91±0.21	1.23±0.52
41	C31	0.78±0.19	0.51±0.07	tr	0.53±0.19
42	11+13-MeC31	1.05±0.35	0.93±0.24	0.80±0.16	0.78±0.35
43	3-MeC31	0.53±0.13	0.52±0.07	tr	0.56±0.17

tr = Trace amount

* Double bond positions were not determined

GC chromatograms obtained from the extractions of adult flies from Day 3 to Day 10 are shown in Figure 5.38. Although the chromatograms of Day 1 and 2 were unknown, the CHC profiles differed significantly between Day 3 and Day 4, while the chemical profiles on Days 4 and 5 were almost similar. This chemical profile was quite different in Day 10 extractions obtained five days later. As can be seen on the chromatograms, the percentage concentration of long-chain CHC compounds in Day 10 adult flies' chemical profiles was significantly reduced compared to the previous days.

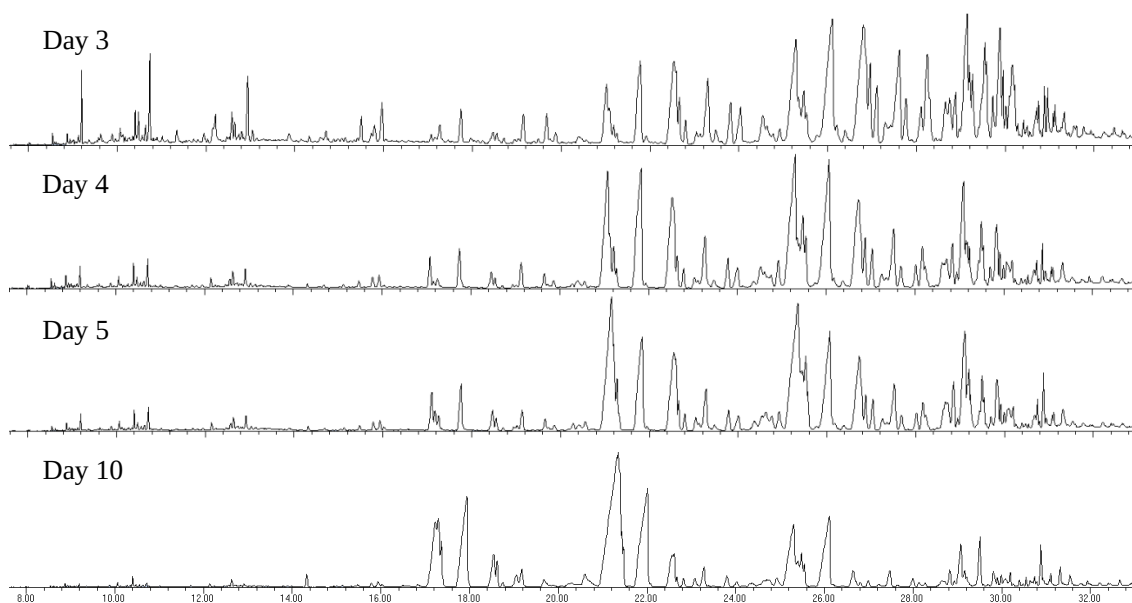


Figure 5.38: GC chromatograms of *C. vicina* outdoor adult flies for Day 3 to Day 10

When the percentage concentrations of n-alkanes obtained in chromatograms were examined (Appendix 27), the percentage concentrations of C23:H and C25:H n-alkanes increased steadily with age, while C27:H and C29:H n-alkanes steadily decreased. C25:H and C27:H chemical profiles were the most abundant n-alkanes. C23:H alkane, whose concentration on Day 3 was a trace amount, increased seven times on Day 10.

The variation of the total percentage concentrations of n-alkanes, branched methyl alkanes and alkenes by age was investigated in Figure 5.39. During the ten days, the predominant group in the chemical profiles was the branched methyl alkanes both in number and concentration. Branched methyl alkane compounds were densely found in the CHC profiles of adult flies extracted on Day 3. In contrast, the percentage concentrations of all CHC compounds were almost similar on Day 10. While the total alkane concentrations were at the same levels on Days 3, 4 and 5, they increased by 3% on Day 10. Day 3 had the highest concentration of branched methyl alkanes. This concentration decreased by 30% with age until Day 10. While it decreased with age, the alkene concentration increased 30 times.

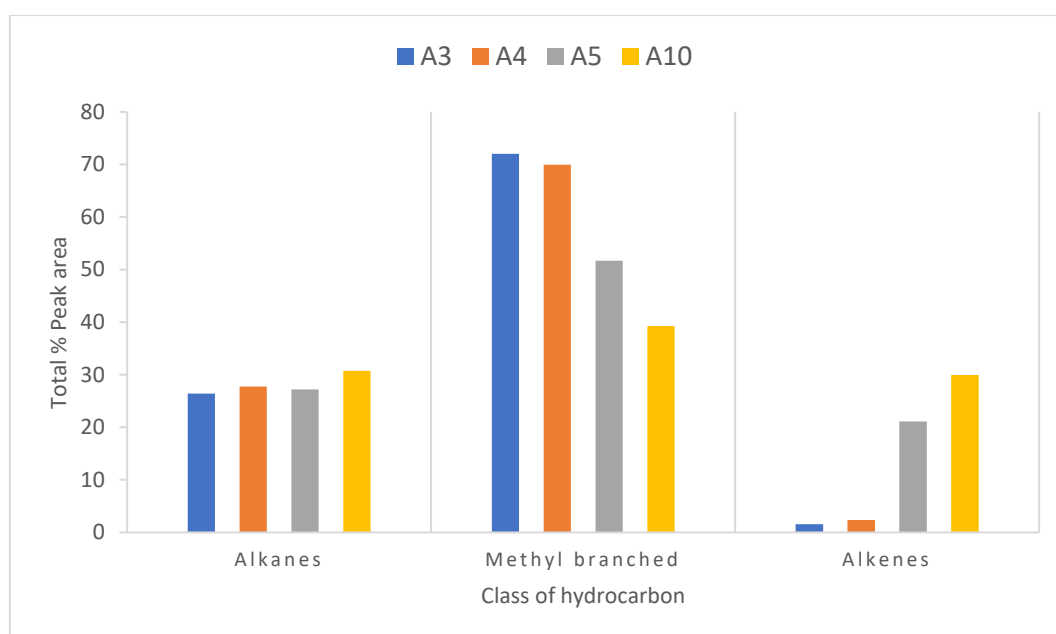


Figure 5.39: Plot showing the total percentage concentration of n-alkanes, branched methyl alkanes and alkenes for *C. vicina* outdoor adult flies

The n-alkane, alkene and branched methyl alkane compounds identified from the chromatograms were applied to PCA. The PCA was carried out using six principal components, describing 95.7% of the variation within the dataset, with the first three principal components comprising 69.8, 22.8 and 3.1%, respectively. Figure 5.40 shows the PCA plot of PC3 vs PC2 vs PC4 for data obtained from Days 3, 4, 5 and 10 adult fly extractions of *C. vicina* reared outdoors.

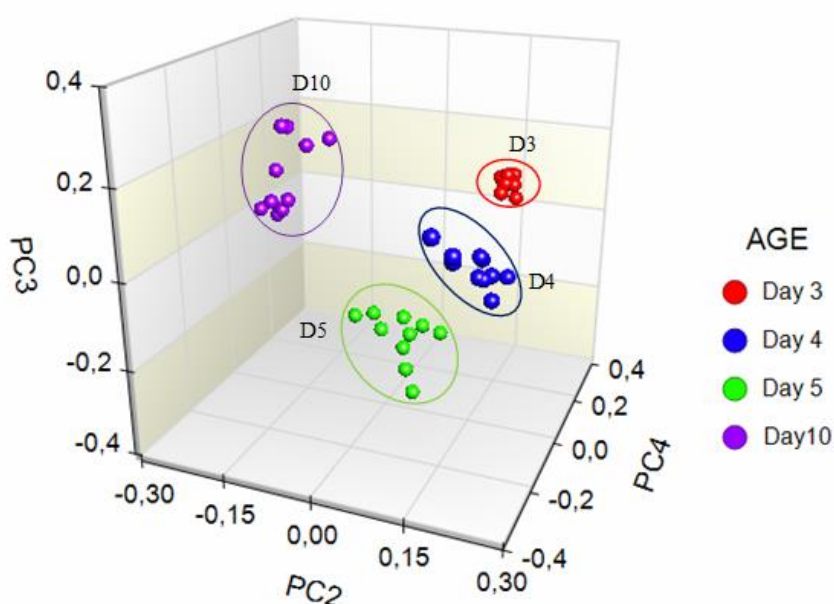


Figure 5.40: PCA 3D plot showing PC3 vs PC2 vs PC4 for *C. vicina* adult flies outdoors, using n-alkanes, alkenes and branched methyl alkanes, with clustering days circled

In this part of the section, age estimation was made for Days 3, 4, 5 and 10 of *C. vicina* outdoor adult flies. The separation of age clusters was clear on the PCA plot and can be used for age estimation accurately. Considering the similarity of Days 4 and 5 chromatograms, PCA separation was quite efficient. The separation of the age clusters shown on the plot was straightforward and followed systematically clockwise. With the CHC analysis and PCA, despite the fluctuating temperature, the results presented for *C. vicina* adult flies offer the potential for age estimation to be established over ten days using CHC analysis and PCA. These results offer a solid foundation for future studies and demonstrate the ability of the method for age estimation based on changes and differences in CHC profiles with the aid of PCA.

5.5.1.2.2 Control group at constant temperature ($25\pm 1^{\circ}\text{C}$)

In order to observe the effect of temperature on the CHC profiles of *C. vicina* adult flies, one group was reared in a cage exposed to fluctuating temperatures in the field and another control group was reared at a constant temperature of $25\pm 1^{\circ}\text{C}$ in the incubator. Thus, the effect of temperature was examined by comparing the changes in the CHC profiles of both adult fly groups. In the chromatograms of *C. vicina* indoor adult flies, a total of 43 CHC compound peaks were obtained from Day 1 to Day 10, and the CHCs are given in Table 5.16. The total CHC concentrations of CHC profiles were n-alkanes (18%), alkenes (11%) and branched methyl alkanes (71%) with chain lengths from C₂₃:H to C₃₁:H. The predominant group in concentration and number was branched methyl alkane in the chemical profiles.

Table 5.16: CHC compounds extracted from the *C. vicina* adult flies reared indoors, total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 10
1	C23	0.94±0.35	0.95±0.24	2.83±0.96	3.74±1.22	6.06±1.54	3.01±0.91
2	9-MeC23	tr	tr	0.98±0.51	1.12±0.41	1.46±0.48	0.73±0.26
3	7-MeC23	tr	tr	0.55±0.19	0.52±0.20	0.71±0.27	tr
4	3-MeC23	tr	tr	0.53±0.11	0.51±0.17	0.61±0.19	0.51±0.11
5	3-MeC24	1.52±0.71	4.87±2.23	12.16±5.12	tr	tr	tr
6	Pentacosene*	1.41±1.21	5.09±2.20	4.34±4.70	11.37±8.83	14.30±14.35	5.00±4.29
7	Pentacosene*	tr	0.97±0.83	8.42±9.66	7.23±11.61	7.33±13.68	2.39±1.03
8	C25	3.57±1.61	4.62±1.42	6.41±1.12	8.20±1.69	12.16±2.85	13.18±3.34
9	11+13-MeC25	3.36±1.26	3.97±0.90	3.53±0.91	2.42±1.75	1.94±1.89	3.83±1.38
10	7-MeC25	1.10±0.19	0.82±0.26	0.65±0.24	0.58±0.13	0.53±0.12	tr
11	3-MeC25	1.69±0.65	1.54±0.40	1.12±0.29	1.13±0.42	1.16±0.40	1.05±0.43
12	C26	1.14±0.48	1.02±0.26	0.63±0.15	0.64±0.30	0.77±0.22	0.71±0.14
13	13+15-MeC26	1.31±0.34	1.07±0.38	0.53±0.10	0.55±0.27	0.54±0.17	tr
14	11+13-MeC26	1.11±0.24	0.81±0.18	0.50±0.16	0.63±0.28	tr	tr
15	8-MeC26	0.89±0.58	0.92±0.47	tr	tr	tr	tr
16	4-MeC26	4.20±1.02	8.45±2.41	11.42±2.50	13.88±2.87	tr	tr
17	Heptacosene*	0.91±0.96	7.31±4.20	7.76±4.62	13.70±3.30	12.76±9.37	8.94±6.09
18	Heptacosene*	tr	1.79±0.66	3.04±4.68	2.47±0.62	3.42±1.76	7.33±4.20
19	C27	9.43±0.90	9.63±2.40	7.41±1.76	7.71±2.52	11.48±3.25	18.34±4.38
20	11+13-MeC27	10.81±0.86	8.44±3.27	4.72±1.50	3.50±1.29	3.00±1.25	3.36±0.99
21	7-MeC27	2.07±0.41	1.62±0.46	0.76±0.28	0.64±0.25	0.52±0.19	0.57±0.14

Peak No.	Peak ID	DAY 1	DAY 2	DAY 3	DAY 4	DAY 5	DAY 10
22	6-MeC27	1.08±1.01	1.10±0.64	0.57±0.25	tr	tr	tr
23	5-MeC27	1.93±0.41	1.53±0.54	0.67±0.21	0.55±0.30	0.50±0.10	tr
24	4-MeC27	1.13±0.77	0.85±0.48	tr	0.50±0.29	tr	tr
25	3-MeC27	5.57±1.93	3.86±1.28	1.77±0.44	1.44±0.55	1.43±0.35	1.12±0.22
26	C28	1.57±0.74	1.05±0.36	0.58±0.15	0.59±0.41	0.65±0.23	0.55±0.17
27	13+15-MeC28	2.27±0.68	1.74±0.76	0.76±0.26	0.64±0.31	0.56±0.14	tr
28	11+13-MeC28	2.13±0.96	1.27±0.53	0.90±0.73	tr	tr	tr
29	8-MeC28	1.59±1.05	1.22±0.45	0.76±0.57	tr	tr	tr
30	4-MeC28	5.72±4.03	1.80±0.97	1.35±0.64	2.69±0.78	3.45±1.65	tr
31	3-MeC28	2.01±0.97	1.60±0.57	0.88±0.19	0.90±0.32	0.98±0.27	tr
32	Nonacosene*	1.97±0.97	1.42±0.58	3.11±2.04	2.47±1.51	2.55±2.23	9.07±4.93
33	Nonacosene*	tr	tr	tr	tr	tr	7.91±4.73
34	C29	6.64±3.49	4.32±1.65	2.37±0.73	2.18±0.82	2.71±0.81	3.23±0.85
35	13+15-MeC29	1.43±0.75	0.84±0.32	tr	tr	tr	tr
36	11+13 MeC29	6.63±3.32	3.95±1.14	1.96±0.54	1.52±0.56	1.42±0.47	1.19±0.31
37	7-MeC29	1.40±0.25	0.92±0.28	0.52±0.13	tr	tr	0.54±0.09
38	3-MeC29	1.86±0.92	1.57±0.61	0.75±0.22	0.64±0.26	0.57±0.14	0.56±0.09
39	C30	0.71±0.28	0.50±0.22	tr	tr	tr	tr
40	8-MeC30	1.17±0.70	0.77±0.27	0.53±0.23	tr	0.52±0.20	tr
41	4-MeC30	0.99±0.70	0.54±0.21	0.62±0.18	0.83±0.30	1.45±0.43	tr
42	3-MeC30	0.67±0.21	0.53±0.17	tr	tr	tr	2.43±0.66
43	11+13 Me31	0.97±0.44	0.63±0.25	0.56±0.12	0.54±0.21	0.84±0.36	1.07±0.35

tr = Trace amount detected (<0.5%)

* Double bond positions were not determined

GC chromatograms (Figure 5.41) of *C. vicina* adult flies show that the percentage concentration of short-chain CHCs increased from Day 1 to Day 5. In contrast, the percentage concentration of longer chain length CHCs decreased with age. While the peaks seen in the chromatograms were sharper, they became flattered. On Day 10, the chromatogram became different, and it meant that the CHC profiles changed.

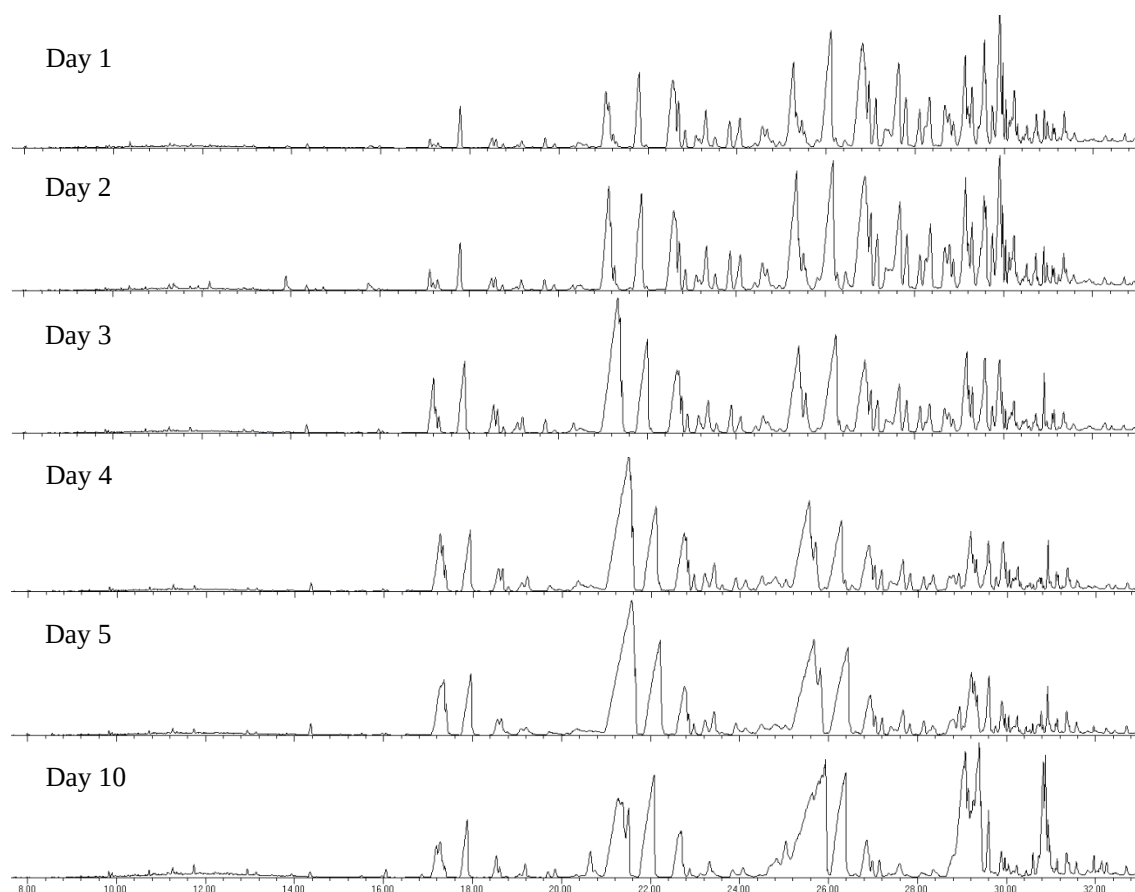


Figure 5.41: GC chromatograms of *C. vicina* indoor adult flies from Day 1 to Day 10

In Figure 5.42, the total concentrations of CHC compounds by classes were plotted against age. According to the plot, although there was a slight decrease in n-alkane concentration from Day 1 to Day 3, it increased from Day 4 onwards. Compared with the previous days, there was an increase of 10% in the percentage concentration of n-alkanes detected on Day 5 and 15% on Day 10. While 70% branched methyl alkanes covered a large area in the adult fly chemical profiles extracted on Day 1, just after the eclosion, this situation decreased with age, and this rate decreased to 18% in Day 10 extractions. On the other hand, while the alkene concentration detected in Day 1 adult flies was 4%, it increased ten times in ten days to constitute 40% of the CHCs.

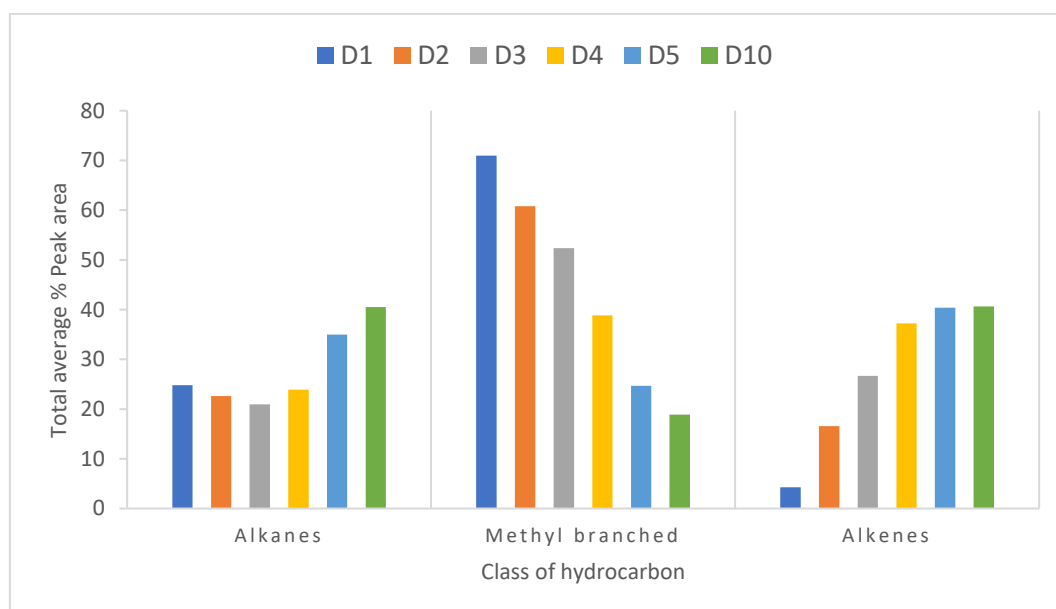


Figure 5.42: Plot showing the total percentage peak area of n-alkanes, branched methyl alkanes and alkenes of *C. vicina* indoor adult flies, from Day 1 to Day 10

Unfortunately, the CHC profiles of the outdoor adult flies could not be extracted for Day 1 and Day 2. Thus, Day 5 and Day 10 samples (the days of the significant changes in the chromatograms) were compared to determine the temperature effect. The total concentrations of CHC compounds in the CHC profiles of in/outdoor adult flies are given in Appendix 28 for Day 5 and Day 10. Also, the total CHC concentration obtained from these samples was shown as the total. The total concentration of n-alkanes detected on Day 5 increased by 21%, and the total alkene concentration increased by 31% over the

past five days. When looking at the change in the CHC concentration of indoor adult flies, the total concentration of n-alkanes increased by 7%. In comparison, the total concentration of branched methyl alkanes decreased by 14%. There was no change in the percentage concentration of total alkenes. It was found that n-alkanes and alkenes were in higher concentration in the chemical profiles of indoor adult flies. The density of branched methyl alkanes was higher in adult flies outdoors.

Considering similarities between the chromatograms of the *C. vicina* indoor adult flies, the difficulty of ageing is understood using only the chromatograms. On the other hand, classifying differences in CHC profiles with PCA allows age estimation easily and faster. The n-alkanes, alkenes and branched methyl alkanes determined in their chromatograms were applied to PCA. The PCA was carried out for *C. vicina* adult flies reared at $25\pm 1^\circ\text{C}$, using six principal components, describing 89.6% of the dataset variation. The first three principal components comprise 69.2, 14.8 and 5.6%, respectively. In Figure 5.43, PC2, PC3 and PC4 were used to plot the relevant scores.

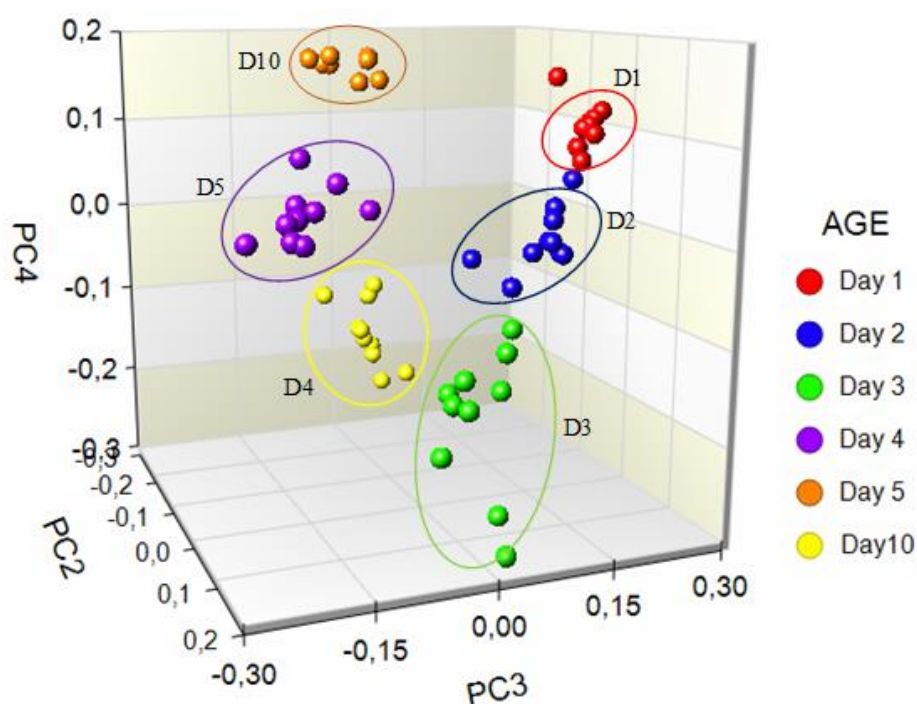


Figure 5.43: PCA 3D plot showing PC4 vs PC2 vs PC3 for *C. vicina* indoor adult flies, using n-alkanes, alkenes and branched methyl alkanes, with clustering days circled

The PCA plot shows that the separation between age clusters is clear between age clusters and can be followed in a systematic clockwise direction. Also, the chemical change between ages can be followed clockwise. These results obtained in *C. vicina* adult flies reared at constant temperature for ten days and CHC profiles extracted are promising for future studies and demonstrate the ability of the method for age estimation, especially in the first five days of age, based on changes and differences in CHC profiles with the aid of PCA.

5.5.2 Discussion of Ageing Adult Flies

Observing hundreds of adult flies in an enclosed space with the decayed corpse and empty puparia indicates that most adult flies develop in the corpse. However, not every adult fly at the scene developed there. By comparing the CHC profiles of indoor and outdoor adult flies, as shown in this study, adult flies that come from outdoors or which developed there can be distinguished. Verifying that most adult flies analysed only consist of a particular age group (for example, 5-10 days old) provides excellent PMI_{min} estimation convenience. Including adult flies in the predictable time interval to estimate the PMI_{min} provides gains for forensic entomology. Moreover, this study demonstrates that younger adult flies can be distinguished from older ones. Pechal et al. [143] identified and aged *Cochliomyia macellaria* and *Ch. rufifacies* adult blowfly species after post-emergence using CHC profiles and found that branched methyl alkanes in particular changed significantly with age. Moreover, no gender alkene was detected in young adult profiles because blowfly adults do not mature sexually for the first 48 hours after emergence. Furthermore, CHCs with longer carbon chains are abundant. It has been stated that adult flies must withstand drier environmental conditions, so the cuticle requires more CHCs with a longer carbon chain that offers increased impermeability under less favourable conditions.

Most of the n-alkanes detected in CHC profiles of both species were OLA compounds; ELA compounds were in the minority. Concentrations of C25:H, C27:H and C29:H were notably higher than others. Furthermore, C23:H concentration increased in Day 10 samples. The percentage concentration of C25:H n-alkane increased with age, while the percentage concentration of C29:H n-alkane tended to decrease. Although these results

support that C25:H and C29:H n-alkanes concentrations can be used as an age indicator, making age estimates thanks to PCA, grouping the differences in all CHC profiles gave a more assured result.

The results showed that when compared to the previous life stages of *L. sericata* and *C. vicina* species, the adult fly stage was the only one in which branched methyl alkanes were the predominant group in the extracted CHC profiles. However, it has decreased with age. The percentage concentrations of n-alkanes tended to increase with age. In particular, the percentage concentration of shorter chain CHCs increased with age. Generally, Day 10 was separated from other age clusters based on their CHC profiles. A decrease in the percentage concentration of shorter chain length CHCs with age was observed only in adult flies.

When the temperature data of *L. sericata* adult flies reared outdoors were examined (Appendix 3), it was seen that the temperatures they were exposed to increased day by day. A reason for the increased n-alkane concentrations in the CHC profiles of Day 10 adult flies could be considered to be temperature. However, a similar situation was observed in Day 10 extractions of adult flies reared at a constant temperature. While the predominant group was branched methyl alkanes in the first 5 days in both sample sets, the predominant group was n-alkanes in Day 10 samples. In this study, the comparison of the CHC profiles of both sample groups showed that the reason for this change might be the temperature effect as well as the species-specific CHC profile.

Adult flies of *C. vicina* were also examined to determine whether the decrease in the branched methyl alkane concentration and the increase in the n-alkane concentration in the Day 10 samples changed depending on age, species, or temperature. A similar situation was observed in CHC profiles of *C. vicina* adult flies reared both indoors and outdoors. Moreover, when the outdoor temperatures of *C. vicina* adult flies were evaluated (Appendix 6), it was understood that they were exposed to similar temperatures for almost 10 days. In this context, it has been understood that this change in CHC profiles of Day 10 adult flies occurred depending on age, independent of species and temperature.

Another age indicator that stood out in adult flies was the density of alkenes in the chemical profiles. Alkenes, which were ignored due to their lower concentrations in

previous life stages, were relatively higher in the adult fly stage, and their concentration tended to increase with age. While the total concentration of CHCs was higher in samples exposed to fluctuating temperatures in larvae and pupae life stages, this situation reversed in adult flies. The total concentration of CHCs in the chemical profiles of adult flies reared under constant temperature was higher, except for the branched methyl alkanes. In contrast, in the chemical profiles of adult flies exposed to fluctuating temperature, the percentage concentrations of branched methyl alkanes were higher, while the n-alkane concentrations were lower. Although this situation shows that temperature affects the cuticle chemical profile, more studies are needed to categorize this effect.

Amendt et al. [160] examined the efficacy of methods for estimating the age of adult flies accurately by analysing ovarian development, the quantity of pteridine in the eyes, the degree of wing damage, CHC profiles and the increasing number of growth layers in the cuticula. They also used age-specific molecular profiles based on gene and protein expression levels and near-infrared spectroscopy and considered the forensic significance of these methods. They stated that pteridine, CHC profile analysis, assessment of cuticular bands and ovarian development methods are the most promising solutions due to their lower cost and ease of use, as well as the fact that different body parts of the same sample may be utilised independently.

With adult flies, when using all of the compounds identified as CHCs from the peaks detected in the chromatograms while applying PCA, using three of six main PCA components, a significant separation was obtained between the clusters. When all compounds are used, PCA groups the compound concentrations change, creating a systematic direction on the PCA plots. In the displayed results, this mainly was clockwise but was occasionally anticlockwise.

In an indoor crime scene investigation, the adult flies that first develop on the corpse will age there as they have difficulty leaving the room. Their indiscernibility when a second generation grows up will be a problem for PM_{Imin} estimations. Different approaches are used for ageing adult flies. However, these are often time-consuming, inconclusive, and of limited use [26]. Age estimates with CHC analysis and PCA were successfully performed to the day in the first five and ten days after the eclosion of *C. vicina*, and *L. sericata* adult flies reared both indoors and outdoors in this investigation. Studies

conducted in both outdoor and indoor areas revealed the impacts of temperature on CHC profiles. The results obtained in this respect, identifying differences between indoor and outdoor samples, eliminate a significant deficiency in the field.

5.6 Summary of Findings on Ageing Life Stages

This chapter investigated age-related changes in the CHC profiles of *L. sericata* and *C. vicina* using GC-MS. The content of the study was determined from eggs to 10-day-old adult flies. Samples in all life cycle stages were reared outdoors, and the temperature effect on the chemical profiles was determined with studies conducted outdoors and indoors. The results offer promising outcomes for forensic investigations into determining PMI_{min}. Changes in CHC profiles of *C. vicina* and *L. sericata* larvae reared under constant temperature were consistent with previous studies. With age, the percentage concentration of short carbon-chain CHCs was taken up by longer chain-length CHCs with higher boiling points. Additionally, larvae reared outdoors under fluctuating temperatures exhibited changes corresponding with the temperature data. It was found that outdoor larvae had more CHC compounds for both species. According to the changes in CHC compounds over age, larval ageing results were obtained when PCA data were plotted in 3D.

The number of CHCs detected in the puparial stage was relatively low. As in the larval stage, the percentage concentration of long-chain CHCs increased with age, and it was almost impossible to determine age according to the change in chromatograms. This study was based on changes in the percentage concentration of branched methyl alkanes, which gave a more distinct separation between the clusters. When only branched methyl alkane data were applied to PCA, pupae were aged at the puparial stage, which was very difficult to achieve in previous studies with 2D plots. These results are the cornerstone of ageing analysis and the use of pupae in criminal cases.

The results obtained from the empty puparia of *L. sericata* are essential in revealing the effects on the weathering time by burying the samples under the soil and keeping them at a constant temperature. While the compounds in the CHC profiles of the samples reared under the soil for 90 days decreased, there was a contemporaneous increase among the

samples reared indoors. These results are significant in terms of defining the crime scene. Furthermore, being able to see the effects of weathering in *C. vicina* empty puparia even in a short period of ten days has revealed the advantages of age estimation with the CHC analysis.

While the percentage concentration of long-chain CHCs increased with age in the larval and puparial stages, this situation was reversed in adult flies. There has been an increase in the percentage concentration of short-chain CHCs with age, which proved critical for age estimation clues for Day 10 adult flies. The chromatograms of the CHC extractions in both the outdoor and indoor CHC extractions of adult flies species were quite similar, but ageing *L. sericata* and *C. vicina* adult flies with CHC within ten days is highly efficient, in that this ageing period indicates even the first five days of ageing.

These research show that the chemical profile has great potential to determine larval and puparial ages and can be used simultaneously for PMI_{min} estimation in forensic investigations. Also, CHC analysis enables young adults to be distinguished from the elderly, whereby differentiation can be determined between adult flies present on a corpse just after eclosion and those attracted by the cadaver's odour.

Chapter 6

Comparative Studies of CHC Profiles of Eggs, Larvae and Pupae for the UK and German Populations

6.1 Introduction

Determining the place where the crime was committed is essential in forensic investigations. If the evidence shows that the place of death and where the corpse was found are different, a simulation carcass can be placed in the corpse discovery place to collect adult flies from that geographical area. Subsequently, the CHC profiles of blowflies collected from carcass and corpses are compared, and differences between CHC profiles provide substantive evidence of whether a body was relocated after death [166]. The ability to distinguish the geographical populations of blowflies by analysis of CHC profiles holds an essential role in the elucidation of forensic cases. Even though there are further studies about how geographical differences affect the succession pattern, it is unclear whether the latter also affects the chemical profile of the insects.

The cuticle layer provides various physiological and ecological functions to insects. The primary role of surface lipids is to reduce water loss and protect living organisms from desiccation and functional importance in terms of stress tolerance, social interactions and mating dynamics [167]. Therefore, specific properties of the cuticle layer such as lipid content, amount, composition, molecular size (chain length), degree of saturation and branching determine the effectiveness of this barrier [168]. Also, different life stages of a species living in different habitats will often have different CHC compositions, as they will need water protection at different water levels [166,169]. Therefore, these characteristics differ between species living in different habitats [170].

Kamhawi et al. [171] identified two distinct populations of *Phlebotomus ariasi* in the south of France using discriminant analysis with GLC. The distance between areas was 900 m. The samples were divided into six tubes labelled A-F analysis. Analysts were blinded to the locations of the samples to facilitate a determination by discriminant and statistical analysis. The results showed similarities between A, D and F tubes (Group 1) and B and C tubes (Group 2). Tube E contained an equal number of individuals from both groups, which means tube E was a mixed sample. At the end of the research, the analysis results were entirely consistent with the statements of the sample collectors. These results proved the value of CHC analysis in the discrimination of populations.

Previous studies showed that CHC analysis is capable of detecting significant differences in the cuticles of different populations and different developmental stages [85,115]. With CHC analysis, it is possible to distinguish between different populations and determine the ages of individuals collected at the crime scene. In this way, CHC analysis can assist forensic investigations in estimating PMI_{min} and determining where the crime occurred and whether the body was relocated. However, basic studies are often carried on under a controlled laboratory regimen. Catts [172] stated that this situation makes interpolation questionable due to missing field data, and controlled field studies need to be developed to improve the accuracy of interpolations.

This study aimed to analyse the CHC profiles of species collected and reared in the UK and Germany using the GC-MS to determine the differences between habitats. This study was carried out in the field instead of in a laboratory environment. CHC profiles of *C. vicina* and *L. sericata* species were extracted from individual samples from eggs to pupae stages reared in two geographical regions: Swindon (UK) and Frankfurt (Germany). Experiments were conducted during the summer season of 2019. The distance between the UK and German populations was over 740 km (Figure 6.1). The results obtained are presented using PCA multivariate statistical analysis to aid in interpreting results and show characteristic differences in chromatograms.

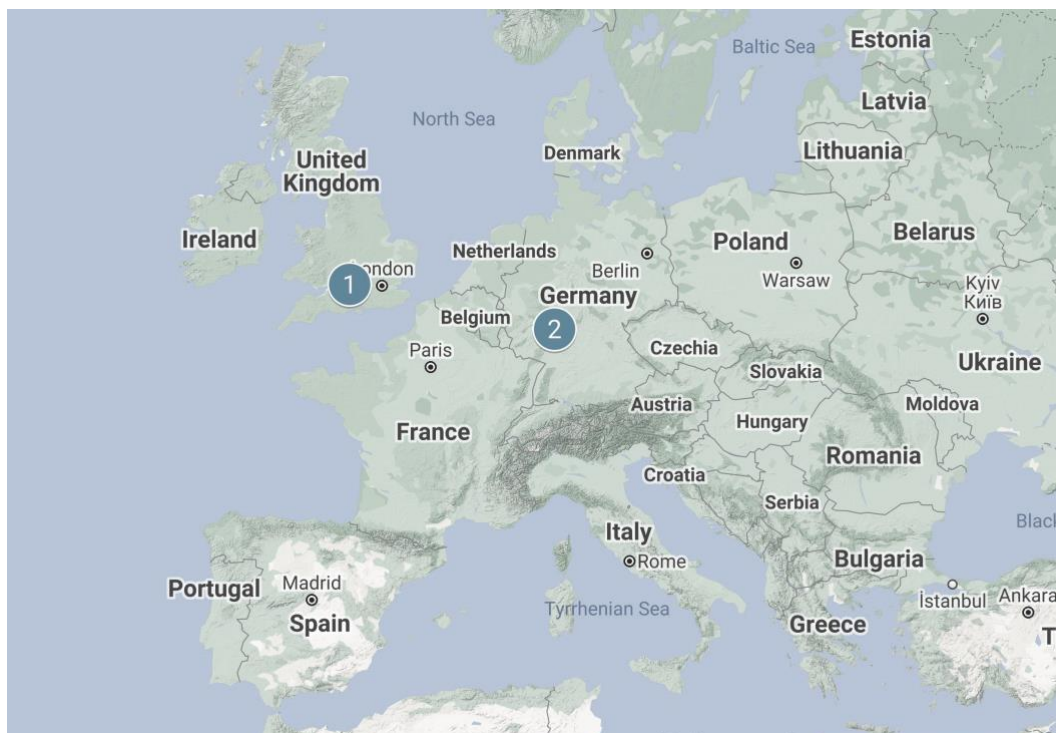


Figure 6.1: Locations of collection sites for populations of *C. vicina* and *L. sericata* species

6.2 Egg Stage

Once female Calliphoridae blowflies reach the corpse, they lay their eggs in natural openings such as the eyes, ears, nose, genitals, or any open wounds that may be present. Grassberger and Reiter [173] examined the immature development times of *Protophormia terraenovae* blowflies in the laboratory at five different constant temperatures of 15, 20, 25, 30 and 35°C. They found that the development times varied inversely with the temperature and noted that it is possible that the developmental time from ovulation to adult eclosion is different in various regions of the world and that the thermal constant of the species is not the same everywhere. Studies characterising variation between geographically different populations of the same species are of great value for future forensic entomological case studies.

The differences in the CHC profiles of blowfly eggs collected from the corpse and those collected from the local area can indicate whether a corpse has been moved, which can quickly clear doubts about the crime scene and provide valuable information to assist

police investigations. Howard and Blomquist [174] reported that in addition to being species-specific, geographically separated populations could exhibit qualitative and quantitative differences in their chemical profiles depending on environmental conditions as well as genetic factors. Therefore, similarities and differences in CHC profiles can be used to indicate the geographical origin of populations.

Although differentiation studies have explored other stages of the life cycle, no research was found in which geographically differences were determined neither based on the morphological characters nor CHC profiles of blowfly eggs. In this respect, this study has undertaken a pioneering mission in the field of forensic entomology by investigating the effects of different habitats and populations on the CHC profiles of the blowfly eggs obtained from wild *C. vicina* and *L. sericata* female flies collected from the UK and Germany. The blowfly eggs were extracted in hexane, and then their chemical profiles were examined by GC-MS. By applying multivariate statistical analysis to the results, the distinction between species was obtained.

6.2.1 Results for Egg Stage

6.2.1.1 *C. vicina*

The eggs of female *C. vicina* adult flies were collected from the wild in the UK and German populations. First, their CHC profiles were extracted using the primary liquid extraction method. Subsequently, chemical profiles were obtained using GC-MS. The CHCs detected in the chromatograms are given in Table 6.1, with the total percentage and standard deviation of each compound. The chain lengths of the detected CHCs range from C22:H to C34:H. The data obtained were used for the subsequent PCA analysis. The compounds with lower concentrations (<0.5%) were not included.

Table 6.1: Compounds extracted from *C. vicina* blowfly eggs collected from the UK and Germany, with the total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	<i>C. vicina</i> (UK)	<i>C. vicina</i> (DE)
1	3-MeC22	ND	0.66±0.26
2	C23	1.06±0.86	1.02±0.37
3	C24	ND	0.50±0.06
4	3-MeC24	ND	6.69±2.49
5	C25	5.61±3.86	5.82±1.69
6	11+13-MeC25	ND	2.78±1.25
7	3-MeC25	ND	0.54±0.19
8	C26	1.30±0.53	0.84±0.18
9	4-MeC26	3.83±2.64	ND
10	Heptacosene*	ND	1.06±0.73
11	C27	18.41±11.99	13.16±2.99
12	11+13-MeC27	1.42±1.06	2.13±0.70
13	3-MeC27	1.59±1.10	1.30±0.50
14	C28	3.11±1.99	2.58±0.78
15	11+13-MeC28	ND	0.61±0.17
16	4-MeC28	7.95±5.12	0.58±0.16
17	3-MeC28	ND	6.34±2.15
18	C29	14.63±10.30	17.27±4.07
19	11+13-MeC29	11.27±7.89	6.71±1.42
20	7-MeC29	3.15±2.08	2.48±0.74
21	5-MeC29	0.73±0.51	0.54±0.16
22	4-MeC29	1.51±0.83	0.88±0.23
23	3-MeC29	4.07±2.87	1.92±0.67
24	C30	0.87±0.64	0.66±0.33
25	8-MeC30	1.43±0.97	0.64±0.27
26	4-MeC30	3.07±2.24	1.32±0.47
27	C31	0.83±0.67	1.83±0.65
28	13+15-MeC31	1.24±0.84	1.31±0.92
29	11+13-MeC31	ND	2.39±0.53
30	9+11-MeC31	5.19±3.50	6.04±2.75
31	3-MeC31	6.42±4.40	5.49±1.93
32	C32	ND	0.52±0.14
33	7-MeC32	ND	0.56±0.18
34	3-MeC32	ND	0.50±0.16
35	C33	1.30±0.86	1.34±0.58
36	C34	ND	1.30±0.54

ND Not detected

* Double bond positions were not determined

The distribution of alkane, alkene, and branched methyl alkane compounds as numbers and percentages by population is given in Table 6.2. A total of 23 CHC compounds were detected in the chemical profiles of *C. vicina* eggs obtained from the UK population. In contrast, 35 CHC compounds were found in the German population. Although the numbers of n-alkanes and branched methyl alkanes detected were different, the total percentage concentrations of these compounds were similar in the chromatograms of the populations. No alkene compound with sufficient concentration was detected in the UK population. On the other hand, heptacosene with a 1% concentration was detected in the German population.

Table 6.2: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *C. vicina* blowfly eggs collected from the UK and Germany

	<i>C. vicina</i> (UK)		<i>C. vicina</i> (DE)	
	number	Total % peak area	number	Total % peak area
n-alkanes	9	47	12	47
Branched methyl HC	14	53	22	52
Alkenes	-	-	1	1
Total	23	100	35	100

Comparative GC-MS chromatograms of blowfly eggs are given in Figure 6.2. There was a difference in peak areas between chromatograms for CHC with longer carbon chains. This distinction may be because the blowfly eggs were relatively small, and the percentage concentration used for extraction was insufficient. Another difference between the chromatograms was the number of CHC compounds detected in the populations. The number of CHC detected in the German population was higher than in the UK population.

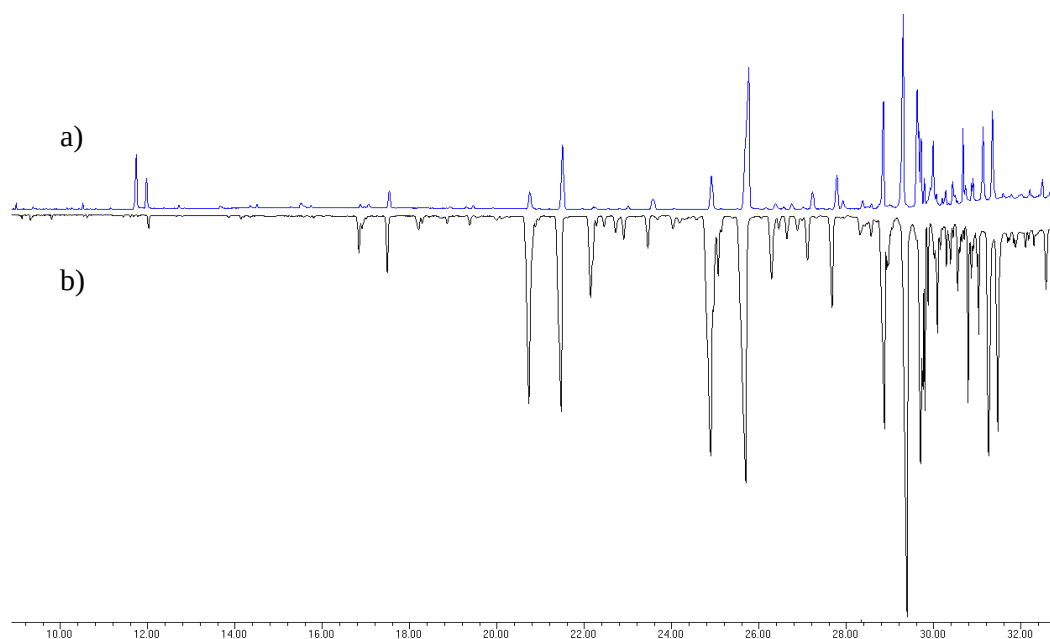


Figure 6.2: GC chromatograms showing CHC profiles of *C. vicina* blowfly eggs collected from a) the UK, b) Germany

The CHC compounds detected in chromatograms can be used as separating markers among populations. C24:H, C32:H and C34:H n-alkanes were detected only in the German population, even at lower concentrations (>0.5%). C25:H, C27:H and C29:H were the most abundant n-alkanes in both populations. In comparison, C27:H was the predominant n-alkane in the UK population and C29:H in the German population. Although the percentage concentrations of CHCs detected in both populations were at a similar level for n-alkanes and branched methyl alkanes, the alkene concentration detected in the German population made a difference; branched methyl alkanes can also be used as separation markers. The differences in their concentrations are given in Appendix 29. 4-MeC26 branched methyl alkane was detected only in the UK population. On the other hand, 3-MeC24, 11+13-MeC25, 3-MeC25, and 11+13-MeC31 branched methyl alkanes were detected only in the German population. Compounds such as 4-MeC28, 11+13-MeC29 and 3-MeC29, which were detected in both populations, had higher concentrations only in the UK population.

Based on the data obtained from the chromatograms, populations could be differentiated by the presence, absence, or concentration of compounds. However, for larger quantities of population or increased numbers of samples, this would be a time-consuming process. For this reason, a statistical separation between populations was provided with the PCA method. The CHC compound data given in Table 6.1 was applied for statistical analysis. PCA was performed using six principal components, describing 99.3% of the dataset variation within the first three, comprising 94.2, 3.7 and 1.4% (PC1, PC2 and PC3), respectively. In Figure 6.3, the plot was formed with the PC2 and PC3 scores, chosen among the six principal components because they clearly showed the variation compared to the others. A data point on the plot represents each sample.

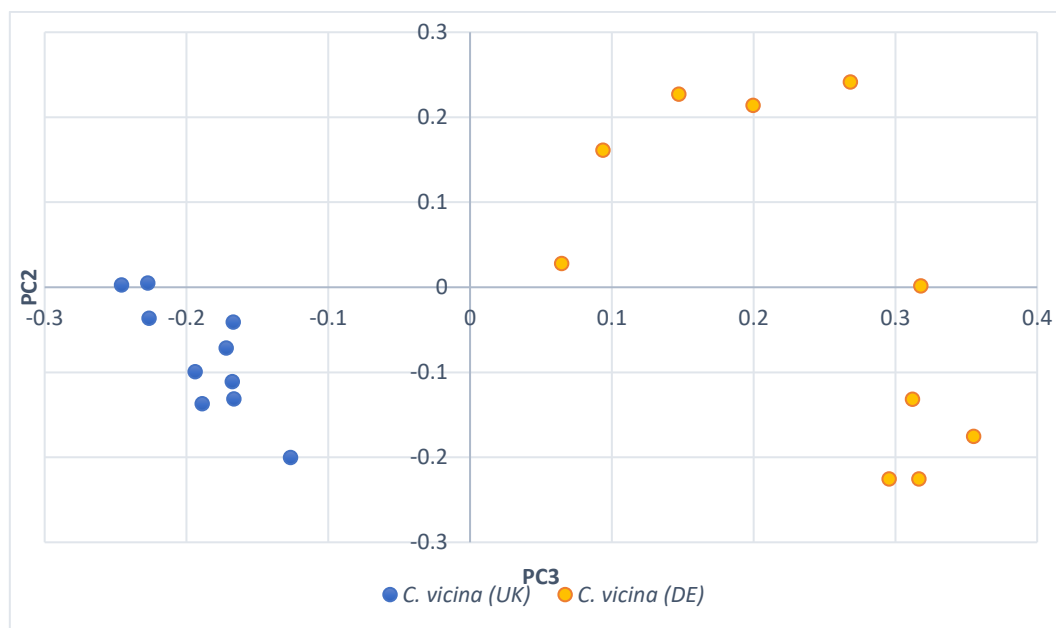


Figure 6.3: PCA plot showing PC3 against PC2 for *C. vicina* blowfly eggs collected from the UK and Germany, using n-alkanes, alkenes and methyl branched alkanes

Discriminant analysis was performed to determine which CHCs were most responsible for separating the UK and German populations. Among all the CHCs given in Table 6.1, the program identified nonacosane (C29:H), 3-methyltetracosane and 4-methyloctacosane as the most critical CHCs in the discriminant analysis.

On the PCA plot, the sample set representing the UK population was on the left, while the sample set representing the German population was on the right side. As can be easily understood from the plot, the distinction between the different populations of *C. vicina* blowfly egg was evident. There was no overlap between populations. The tight clustering of repeated samples in the PCA plot gives information about the variation and reproducibility of the experiment.

6.2.1.2 *L. sericata*

The blowfly egg extractions of *L. sericata* female flies collected from the wild in the UK and Germany were analysed by GC-MS. The CHC compounds detected in the chemical profiles are given in Table 6.3. The chain lengths of the detected CHCs range from C22:H to C33:H. The data obtained were used for the subsequent PCA analysis. CHCs with insufficient concentration were not included (<0.5%).

Table 6.3: Compounds extracted from *L. sericata* blowfly eggs collected from the UK and Germany, with the total percentage of each compound present and percentage standard deviation for each day

Peak No.	Peak ID	<i>L. sericata</i> (UK)	<i>L. sericata</i> (DE)
1	3-MeC22	ND	0.64±0.29
2	Tricosene*	1.28±0.59	0.98±0.58
3	C23	2.32±0.98	1.69±0.98
4	11+13-MeC23	1.04±0.51	1.31±0.64
5	C24	tr	0.52±0.27
6	3-MeC24	ND	0.81±0.35
7	Pentacosene*	5.11±2.03	3.25±1.97
8	C25	3.65±1.25	2.92±1.60
9	11+13-MeC25	2.78±1.22	2.34±1.39
10	C26	0.95±0.17	1.04±0.57
11	3-MeC26	1.01±0.33	2.35±1.35
12	Heptacosene*	4.14±1.95	2.64±1.60
13	C27	13.76±2.93	17.05±9.32
14	11+13-MeC27	1.00±0.33	1.53±0.80
15	3-MeC27	1.49±0.36	2.58±1.41
16	C28	2.50±0.49	2.07±1.24
17	11+13-MeC28	ND	0.66±0.64
18	4-MeC28	1.06±0.21	ND
19	3-MeC28	ND	1.84±1.41
20	Nonacosene*	1.12±0.41	1.51±1.29
21	Nonacosene*	ND	0.87±1.07
22	C29	20.03±4.68	10.39±5.17
23	11+13-MeC29	5.92±2.01	6.78±3.36
24	9-MeC29	2.36±0.75	2.69±1.29

Peak No.	Peak ID	<i>L. sericata</i> (UK)	<i>L. sericata</i> (DE)
25	7-MeC29	2.80±1.22	3.23±2.26
26	6-MeC29	ND	2.28±1.41
27	4-MeC29	0.98±0.37	1.90±1.22
28	3-MeC29	1.21±0.73	ND
29	C30	0.75±0.28	0.77±0.79
30	8-MeC30	0.53±0.20	1.11±1.10
31	4-MeC30	ND	1.76±1.65
32	3-MeC30	1.57±0.66	1.44±1.25
33	Hentriacontene*	4.25±1.64	ND
34	C31	1.93±0.58	2.02±1.64
35	13+15-MeC31	5.50±1.14	5.46±4.00
36	9+11-MeC31	5.38±0.79	6.21±3.81
37	C32	ND	1.11±0.85
38	7-MeC32	0.77±0.13	1.53±0.78
39	5-MeC32	0.63±0.25	0.60±0.52
40	3-MeC32	ND	0.59±0.26
41	C33	1.66±0.35	1.64±0.90

ND Not detected

* Double bond positions were not determined

When the CHCs detected in GC chromatograms were examined as n-alkanes, alkenes and branched methyl alkanes (Table 6.4), 31 and 37 CHC compounds were detected in the UK and German populations, respectively. Despite identifying similar numbers of n-alkanes and alkenes, more branched methyl alkane compounds were detected in the German population. Although only one nonacosene was identified in the chromatograms, it was considered two different alkene compounds due to the double bond location change, whose endpoint was not explicitly determined. Furthermore, the total percentage concentrations of the populations were different. The total concentration of n-alkanes and alkenes in *L. sericata* profiles collected from the UK was 7% higher than the German population; conversely, the total concentration of branched methyl alkanes in the German population was 13% higher than the UK population.

Table 6.4: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *L. sericata* blowfly eggs collected from the UK and Germany

	<i>L. sericata</i> (UK)		<i>L. sericata</i> (DE)	
	number	Total % peak area	number	Total % peak area
n-alkanes	9	48	11	41
Branched methyl HC	17	36	21	50
Alkenes	5	16	5	9
Total	31	100	37	100

Comparing the GC chromatograms (Figure 6.4) showing the CHC profiles of *L. sericata* blowfly eggs collected from the UK and German populations, the differences in the peak areas of the compounds, especially C27:H and C29:H, were remarkable. Furthermore, the peak areas of the same compounds detected in the German population were higher than those detected in the UK.

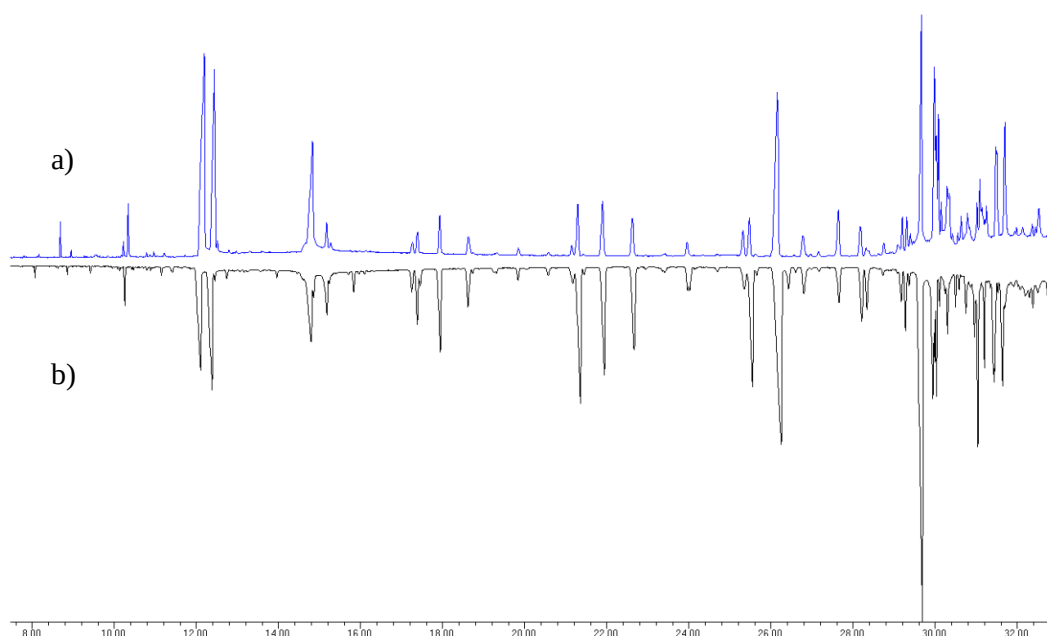


Figure 6.4: GC chromatograms showing CHC profiles of *L. sericata* eggs collected from a) Germany, b) the UK

Except for C32:H, which was not detected in the UK population, other n-alkanes were detected in both populations. Although C25:H, C27:H and C29:H n-alkanes were found in higher concentrations in both populations, C29:H n-alkanes were predominant in the UK population, while C27:H n-alkanes were predominant in the German population. 4-MeC28 and 3-MeC29 were detected only in the UK population. In contrast, 3-MeC24, 11+13-MeC28, 3-MeC28, 6-MeC29 and 4-MeC30 were detected only in the German population. Hentriacontene was detected only in the UK population, and nonacosene was detected only in the German population. Apart from these CHCs, other CHCs given in Table 6.3 were detected in both populations.

Although the populations can be distinguished by the compounds detected in the CHC profiles of the UK and German populations examined, PCA was applied to visualise the results obtained. The CHC compound data given in Table 6.3 was applied for statistical analysis using six principal components, describing 98.4% of the dataset variation within the first three, comprising 93.9, 3.5 and 1.0%, respectively. Since it gave a more apparent separation between the PCs and created harmony between the previous plots, the PCA plot given in Figure 6.5 was created using PC2 and PC3 components. A data point on the plot represents each sample. The cluster representing the UK population is on the left of the PCA plot, while the cluster representing the German population is on the right.

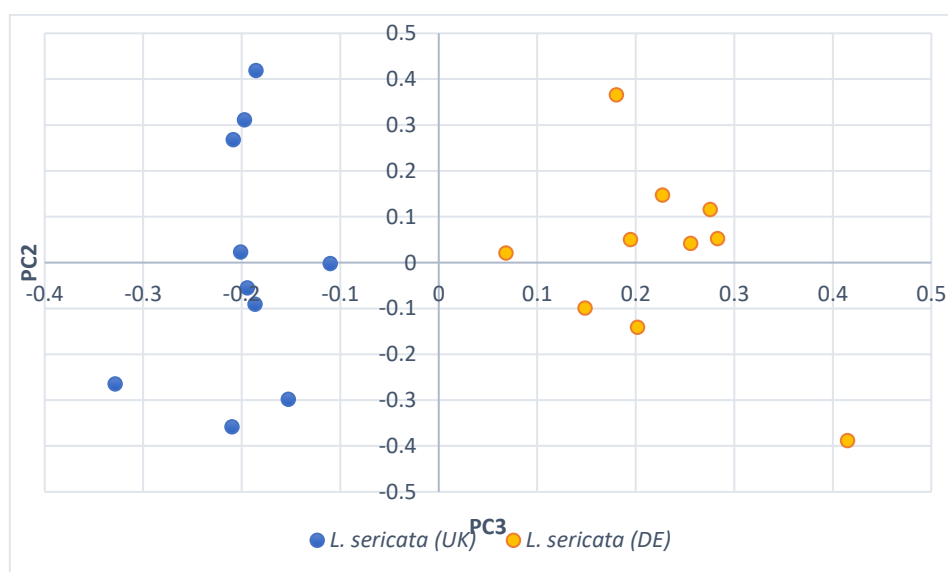


Figure 6.5: PCA plot showing PC3 against PC2 for *L. sericata* blowfly eggs collected from the UK and Germany, using n-alkanes, alkenes and methyl branched alkanes

Discriminant analysis was performed to determine which CHCs were most responsible for separating populations of UK and German *L. sericata* adult fly eggs. Among all the CHCs, the program identified heptacosane (C27:H), nonacosane (C29:H), 13+15-Methynonacosane and pentacosene as the essential CHCs in the discriminant analysis.

The differences in CHC profiles among the *L. sericata* populations analysed reflect geographical diversity. As can be easily understood from the plot, the distinction between the different populations of *L. sericata* blowflies eggs was obvious: there was no overlap between populations. Data was prepared with only two sample sets, which affected scattering. Therefore, the scattered or tight dispersion of the sample sets that form the clusters was ignored in this study.

6.2.2 Discussion of Egg Stage Comparison

Environmental factors such as temperature, photoperiod, developmental stage, sex and genetic differences affect CHC compositions. Waterproofing abilities are related to the structural and physical properties of CHCs, including chain length, unsaturation and methyl branching. Therefore, environmental conditions can be determined by visualising the variations in CHC profiles.

In this study, differences in CHC profiles between the eggs of female adult flies of *C. vicina* and *L. sericata* species collected from the wild in the UK and Germany were detected and visualised using CHC analysis and PCA. It was shown that at an early stage, such as adult fly eggs, using CHC with PCA analyses made it possible to distinguish separate geographical populations for both *L. sericata* and *C. vicina* eggs based on their chemical profiles. Detection of differences even in such small structures makes CHC analysis a powerful method. Moreover, it can quickly result in forensic investigation to determine the crime scene and relocation of a corpse. CHC analysis method can be developed with future studies involving more blowfly eggs species and populations. In this respect, the studies presented in this part are preliminary and promising for future studies.

6.3 Larval Stage

Larvae are the most common life stage in early decomposition at a crime scene. Therefore, they can be used in forensic entomology to obtain information about the crime scene and corpse location, as well as for species identification and age estimation. Insects are poikilothermic, meaning their internal body temperatures fluctuate with changing ambient temperatures [7]. Therefore, the most significant influence on the development is temperature and the development time depends on the ambient temperature. Moreover, many biotic and abiotic factors can affect development, for example, weather, gender, geographical location and population genetics [150]. These factors significantly affect development by increasing or decreasing the time it takes them to complete their life cycle. Therefore, a better understanding of the variability and the factors affecting development is essential to improve evidence collection and analysis techniques.

The cuticle layer acts as a barrier to prevent water loss, which is fatal for larvae. The larval response to water loss at higher temperatures significantly increases CHCs with higher carbon numbers and reduces those with shorter carbon chains, as previously shown in Chapter 5. This spurt has the effect of reducing cuticular permeability and thus water loss. Larval responses to lower temperatures also work in the opposite direction, depending on the temperature change. However, longer chain n-alkanes do not change significantly at lower temperatures. Toolson [89] explained this situation in terms of branching and unsaturation, reducing the strength of the Van der Waal bond between CHCs, increasing their fluidity and increasing their permeability. This response of larval CHC profiles to temperature changes can provide important information about a crime scene.

In this study, the variation between populations of *C. vicina* and *L. sericata* species living in different habitats (the UK and Germany) was determined based on the qualitative and quantitative differences in CHC profiles. Since the CHC composition can be changed with the developmental stage and temperature, the extractions included the beginning and end of the larval developmental stages and examined the temperature and geographical effects. CHC compounds were analysed by GC-MS. By applying multivariate statistical analysis to the results, the distinction between species was obtained.

6.3.1 Results for Larval Stage

6.3.1.1 *C. vicina*

CHC profiles were analysed with GC-MS by extracting *C. vicina* larvae reared in the UK and Germany on the first and last days of the larval period. The larval development period was the same for both populations. The CHCs detected in the chromatograms of these two populations are given in Table 6.5. Detected CHCs such as alkenes were not included as they were not of sufficient concentration (< 0.5%). The chain lengths of the detected CHCs range from C16:H to C34:H. The data obtained were used for the subsequent PCA analysis.

Table 6.5: Compounds extracted from the first day and last day of the larval stages *C. vicina* reared outdoors in the UK and Germany, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>C. vicina</i> (UK) first day	<i>C. vicina</i> (DE) first day	<i>C. vicina</i> (UK) last day	<i>C. vicina</i> (DE) last day
1	C16	3.17±0.59	ND	1.82±0.72	ND
2	C17	1.40±0.40	ND	1.29±0.52	ND
3	7-MeC17	ND	ND	0.98±0.40	ND
4	3-MeC17	1.50±0.61	ND	2.04±0.63	ND
5	Octadecene*	1.50±0.61	ND	2.30±0.79	ND
6	C18	6.89±1.49	1.25±0.38	5.12±1.40	1.18±0.19
7	7-MeC18	3.29±0.97	ND	2.67±0.85	ND
8	C19	3.31±0.75	0.51±0.14	2.18±0.77	0.50±0.06
9	9+11-MeC19	ND	ND	1.71±0.30	ND
10	3-MeC19	2.56±0.33	ND	1.46±0.31	ND
11	C20	8.96±1.37	1.43±0.28	5.36±1.02	1.76±0.21
12	C21	5.56±0.81	1.33±0.25	2.91±0.60	0.68±0.14
13	5-MeC21	2.81±1.13	ND	1.51±0.63	ND
14	3-MeC21	ND	ND	1.62±0.61	ND
15	Docosene*	ND	ND	3.98±0.79	ND
16	C22	12.43±2.08	2.98±0.64	6.50±1.22	2.12±0.25
17	C23	11.62±1.47	4.81±1.50	5.42±0.96	2.14±0.36
18	5-MeC23	ND	ND	2.01±0.74	ND
19	4-MeC23	ND	ND	ND	0.57±0.17

Peak No.	Peak ID	<i>C. vicina</i> (UK) first day	<i>C. vicina</i> (DE) first day	<i>C. vicina</i> (UK) last day	<i>C. vicina</i> (DE) last day
20	3-MeC23	ND	ND	ND	1.11±0.14
21	C24	9.52±1.04	6.94±1.91	4.69±1.68	4.07±0.59
22	3-MeC24	ND	1.41±0.42	ND	0.50±0.10
23	C25	9.13±2.44	7.83±2.29	3.69±0.71	5.33±1.17
24	11+13-MeC25	ND	ND	ND	0.64±0.16
25	4-MeC25	ND	0.82±0.40	ND	0.58±0.10
26	3-MeC25	ND	0.78±0.26	ND	1.10±0.14
27	C26	3.78±0.37	7.97±2.79	2.24±0.40	5.77±0.86
28	4-MeC26	ND	2.15±0.94	ND	0.71±0.23
29	3-MeC26	ND	0.74±0.25	ND	0.51±0.08
30	C27	3.43±1.03	8.56±2.75	9.53±2.40	14.17±4.23
31	11+13-MeC27	ND	ND	ND	0.74±0.10
32	4-MeC27	ND	1.00±0.69	ND	0.55±0.14
33	3-MeC27	ND	1.01±0.62	1.17±0.29	1.98±0.58
34	C28	5.25±1.12	9.08±3.10	5.54±1.48	6.26±1.11
35	4-MeC28	ND	1.05±0.61	ND	0.57±0.15
36	3-MeC28	ND	1.02±0.65	ND	0.51±0.12
37	C29	3.89±1.32	7.14±3.21	13.38±3.97	12.33±2.30
38	7-MeC29	ND	ND	ND	0.84±0.25
39	4-MeC29	ND	1.07±0.74	ND	0.50±0.11
40	3-MeC29	ND	0.55±0.27	0.62±0.13	1.13±0.22
41	C30	ND	5.53±2.99	2.95±1.16	4.70±0.81
42	5-MeC30	ND	2.15±1.37	ND	1.74±0.71
43	4-MeC30	ND	1.51±0.85	ND	1.02±0.18
44	3-MeC30	ND	2.59±2.75	ND	1.65±0.32
45	C31	ND	5.57±2.49	2.68±0.64	6.79±0.67
46	13+15-MeC31	ND	ND	ND	1.43±0.35
47	11+13-MeC31	ND	1.27±0.69	ND	1.17±0.13
48	3-MeC31	ND	0.99±0.46	ND	1.27±0.19
49	C32	ND	4.81±2.43	1.22±0.34	6.23±1.06
50	C33	ND	3.05±1.65	1.38±0.35	5.36±0.69
51	C34	ND	1.18±0.68	ND	0.68±0.16

ND Not detected

* Double bond positions were not determined

In Table 6.6, the distribution of CHCs detected in the chromatograms according to the n-alkane, alkene and branched methyl alkane classification is given. A total of 19 CHCs were detected in the first instar larvae belonging to the UK population, and 33 CHCs were detected in the German population. Approximately similar numbers of n-alkanes were detected in both populations. However, four times more branched methyl alkanes were detected in the German population than in the UK.

Table 6.6: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *C. vicina* first instar larvae reared outdoors in UK and Germany

	<i>C. vicina</i> (UK) first day		<i>C. vicina</i> (DE) first day	
	number	Total % peak area	number	Total % peak area
n-alkanes	14	88	17	80
Branched methyl HC	4	10	16	20
Alkenes	1	2	-	-
Total	19	100	33	100

Comparative GC chromatograms of *C. vicina* first instar larvae of the UK and German populations are given in Figure 6.6. The remarkable difference in the chromatograms was that while CHCs with shorter chain lengths were found in higher concentrations in the UK population, CHCs with longer carbon chain lengths were higher in the German population.

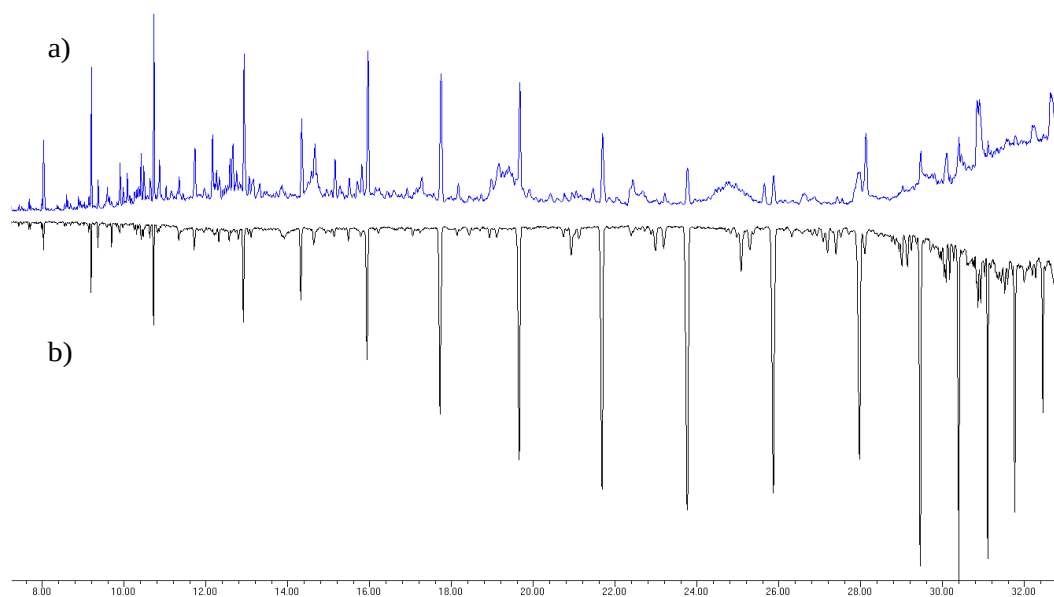


Figure 6.6: GC chromatograms showing CHC profiles of *C. vicina* first instar larvae reared in a) the UK, b) Germany

When the percentage concentrations of n-alkanes detected were examined in detail (Figure 6.7), it was found that C22:H and C23:H n-alkanes were abundant in the profile of the UK population. In contrast, C27:H and C28:H n-alkanes were abundant in the German population. Also, the percentage concentrations of n-alkanes were higher than in the German population. When the branched methyl alkanes were examined, 3-MeC17, 7-MeC18, 3-MeC19 and 5-MeC21 branched methyl alkanes were detected in only the UK population. Other branched methyl alkanes given in Table 6.5 were detected only in the German population. There was no common branched methyl alkane detected in both profiles. Octadecene was detected only in the UK population, even at a 1% concentration. Since the other alkenes' detected concentrations were below 0.5%, they were not included for both populations.

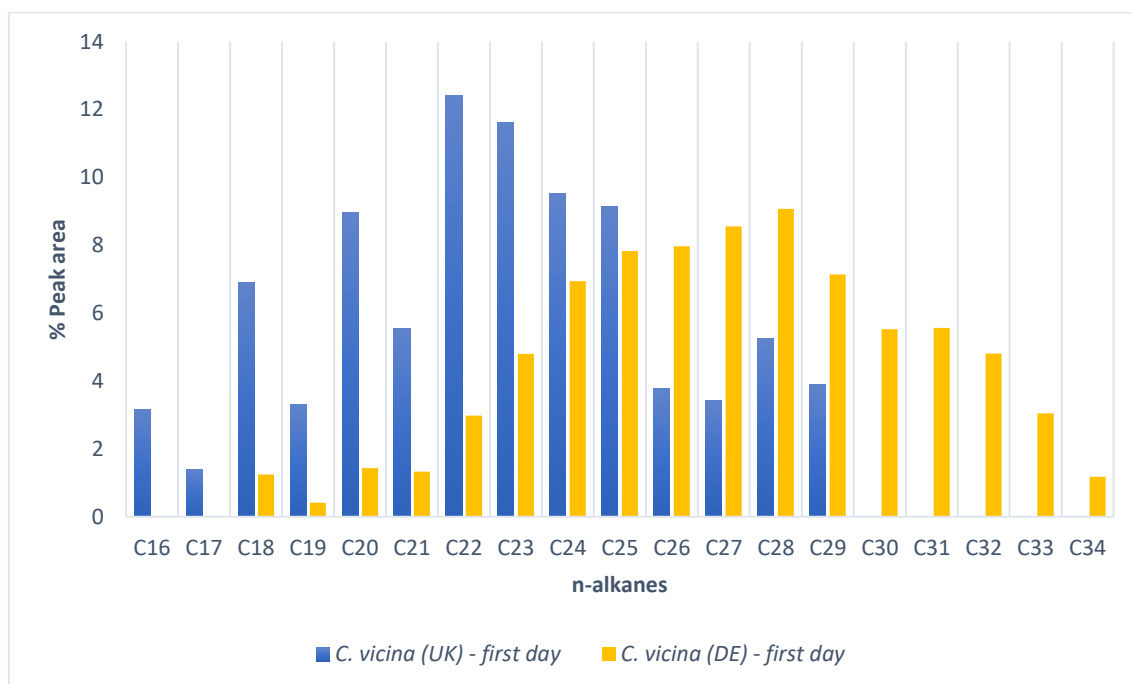


Figure 6.7: Plot showing the percentage concentrations of n-alkanes detected in *C. vicina* first instar larvae reared outdoors in the UK and Germany

C. vicina larvae were reared during the larval stage in outdoor cages exposed to ambient temperatures in both the UK and Germany. When the temperature data of *C. vicina* larval stages given in Appendixes 4 and 9 were examined, it was seen that the larvae reared in the UK were exposed to an average temperature of 19.6°C on the first day, and the larvae

reared in Germany were exposed to an average temperature of 18.8°C. The comparative temperature data for the same period is also given in Appendix 30, indicating that the UK population was exposed to a higher temperature during the first instar larvae stage than the German population; however, the German population was exposed to a higher maximum temperature during the egg stage. This situation explains the distribution of CHCs in the larval structure.

When the CHC profiles of *C. vicina* post-feeding larvae on the last larvae day were observed (Figure 6.8), it was found that the shorter carbon chain CHCs were abundant in the post-feeding larvae reared in the UK. In contrast, the longer carbon chain CHCs were abundant in those reared in Germany. In addition, when the peak areas of CHCs detected were compared, those detected in larvae reared in Germany were higher than in the UK.

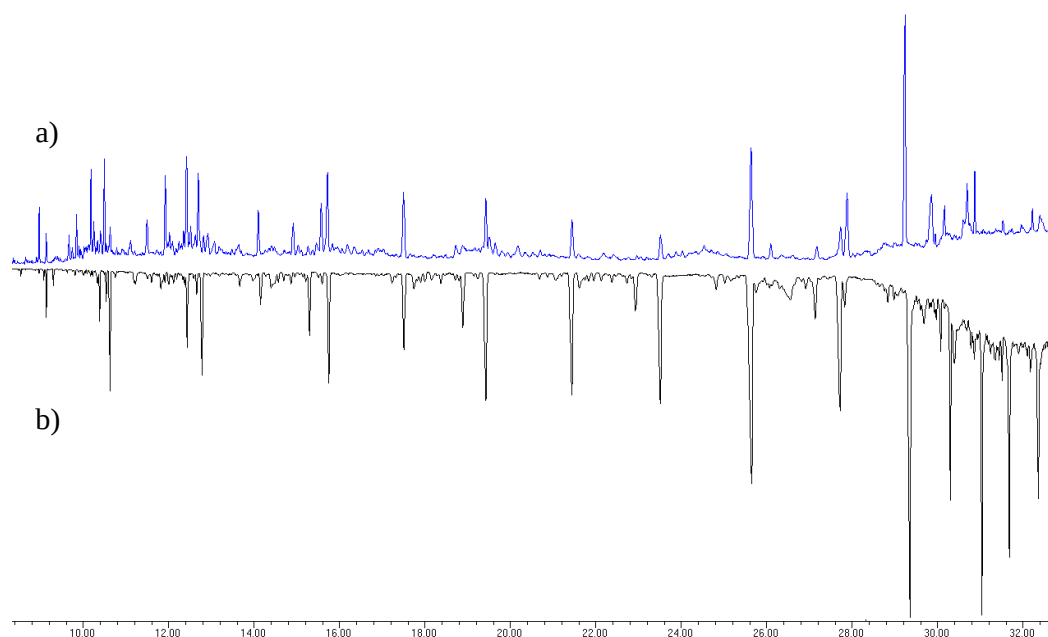


Figure 6.8: GC chromatograms showing CHC profiles of *C. vicina* post-feeding larvae reared in a) the UK, b) Germany

The numbers of CHCs determined in the chromatograms are given in Table 6.7 according to the classification of CHC. While a total of 30 CHCs were detected in the larvae on the last day of the larval stage reared in the UK, 39 were for those reared in Germany. In both populations, there were similar numbers of n-alkanes but at different concentrations. The number of branched methyl alkanes detected in the German population was almost twice that of the UK population.

Table 6.7: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *C. vicina* post-feeding larvae reared outdoors in UK and Germany

	<i>C. vicina</i> (UK) last day		<i>C. vicina</i> (DE) last day	
	number	Total % peak area	number	Total % peak area
n-alkanes	18	78	17	80
Branched methyl HC	10	16	22	20
Alkenes	2	6	-	-
Total	30	100	39	100

Figure 6.9 shows the percentage concentrations of n-alkanes detected in post-feeding larvae reared in the UK and Germany. Concentrations of n-alkanes from C16:H to C24:H were higher in the UK population, while concentrations of n-alkanes from C25:H to C33:H were higher in the German population. C29:H n-alkane was the predominant compound in the UK population, and its concentration was higher than the German population. The predominant compound in the German population was C27:H n-alkane. The German population contained 2% more compounds than the UK population as a total percentage of n-alkane concentration and 4% more as a total percentage of branched methyl alkane concentration. Branched methyl alkanes of C17:H to C21:H and their isomers were detected only in the UK population. Branched methyl alkanes of C23:H and C31:H and their isomers were detected only in the German population. The branched methyl alkanes that were commonly detected in both populations were 3-MeC27 and 3-MeC29, and their concentration in the German population was higher than in the UK population. Octadecene and docosene were alkenes detected in the UK population,

accounting for 6% of the total CHC concentration. Alkenes were omitted since the percentage concentration of alkenes detected in the German population was below 0.5%.

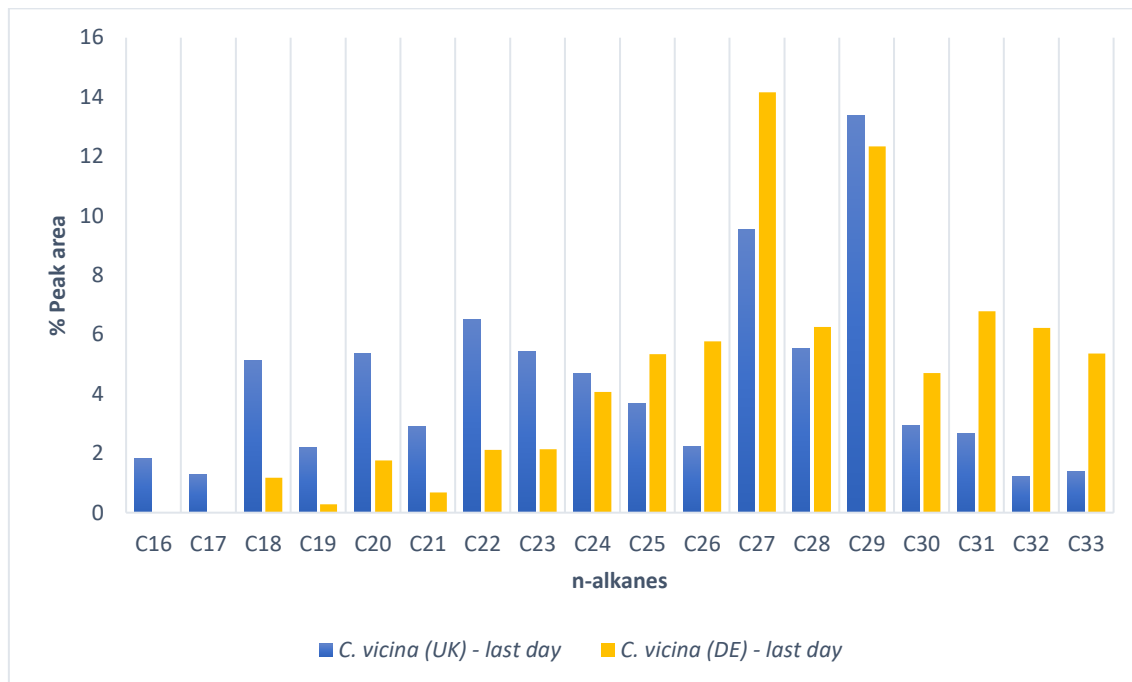


Figure 6.9: Plot showing the percentage concentrations of n-alkanes detected in *C. vicina* post-feeding larvae reared outdoors in the UK and Germany

The temperature plot given in Appendix 30 shows that the higher temperatures decreased towards the end of the larval stages of *C. vicina* larvae reared in the UK; in particular, the maximum temperature during the day dropped from 40°C to 20°C. Towards the end of the larval stage reared in Germany, the opposite situation occurred, with increasing day and night temperatures. The changes in CHC compounds of the larvae seem to be in harmony with the temperature changes. It was an expected result that CHCs with a longer carbon chain and a higher boiling point would be denser concentrations in the chemical profiles of the larvae exposed to higher temperatures. In contrast, CHCs with a shorter carbon chain and lower boiling point in the chemical profiles of the larvae reared at lower temperatures were more intense.

The data given in Table 6.5 were applied to PCA to visualise the differences and discriminate according to this difference between the CHC profiles of the samples taken from the UK and German populations on the first day and the last day of the *C. vicina* larval period. PCA was performed using six principal components, describing 96.8% of the dataset variation, with the first three principal components being 75.7, 16.2 and 4.9%, respectively. 3D plots were used to clearly visualise the separation between clusters. In order to create the PCA plot given in Figure 6.10, PC2, PC3 and PC3 scores were preferred to achieve clearer separation between the clusters.

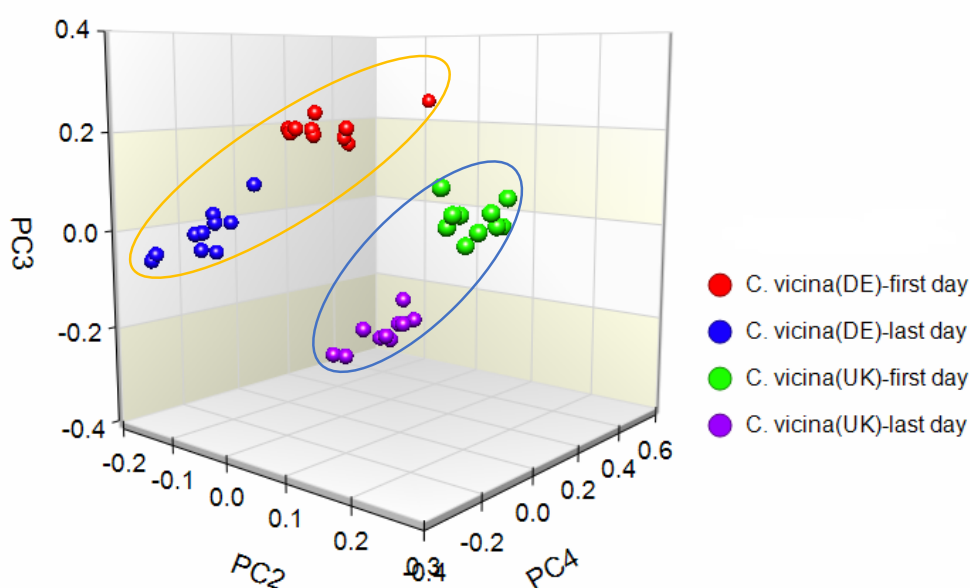


Figure 6.10: PCA plot showing PC3 vs PC2 vs PC4 for *C. vicina* larvae reared outdoors in the UK and Germany, using n-alkanes, alkenes and methyl branched alkanes, clusters were grouped by populations

Discriminant analysis was performed to determine which CHCs were most responsible for separating populations of UK and German *C. vicina* first instar and post-feeding larvae. Among all the CHCs (Table 6.5), the program identified docosane (C22:H), pentacosane (C25:H), heptacosane (C27:H) and nonacosane (C29:H) as the most important CHCs in the discriminant analysis.

The PCA plot indicates that the UK and the German populations were separated from each other both on the first day and last day of the larval period. Clusters representing larvae reared in the UK gave positive values on the PC2 axis, while clusters representing larvae reared in Germany gave negative values. There was no overlap between populations. The tight distribution of the repeat samples forming the clusters on the plot also showed that the variation and reproducibility of the results were high. Chapter 5 showed that the age estimation of the larvae during the larval stages could be undertaken daily with CHC analysis, and the results presented here showed changes in CHC profiles with temperature based on developmental stage and age.

6.3.1.2 *L. sericata*

The CHC profiles of *L. sericata* larvae obtained from the populations reared outdoors in the UK and Germany were extracted on the first day and the last day of the larval period (just before the puparial period) in order to observe the difference between the populations. The CHC compounds detected in the chemical profiles are given in Table 6.8. The chain lengths of the detected CHCs ranged from C16:H to C33:H. In the CHC profiles, n-alkane and branched methyl alkanes were detected intensively. Plenty of the alkenes were not included because of their lower concentration (< 0.5%). The data obtained were used for the subsequent PCA analysis.

The larval period of the larvae reared outdoors in the UK lasted ten days and the larval period of the larvae reared in Germany lasted 11 days. CHC profiles of the first instar larvae hatched from the egg and the larvae extracted on the last larval day just before the pupae stage were examined. Thus, results were obtained independent of the completion of the larval stages at different times.

Table 6.8: Compounds extracted from the first day and last day of the larval stages of *L. sericata* reared outdoors in the UK and Germany, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>L. sericata</i> (UK) first day	<i>L. sericata</i> (DE) first day	<i>L. sericata</i> (UK) last day	<i>L. sericata</i> (DE) last day
1	C16	2.24±0.48	ND	ND	ND
2	C17	1.23±0.34	ND	ND	ND
3	7-MeC17	0.79±0.33	ND	ND	ND
4	3-MeC17	1.81±0.35	ND	0.51±0.18	ND
5	C18	5.59±1.10	1.37±1.26	1.29±0.37	0.50±0.10
6	7-MeC18	2.68±0.64	ND	0.90±0.33	ND
7	C19	3.20±0.82	ND	0.83±0.30	0.50±0.06
8	9+11 MeC19	2.29±1.34	ND	2.60±1.56	ND
9	3-MeC19	1.56±0.58	ND	0.51±0.17	ND
10	Eicosene*	3.90±1.09	ND	1.03±0.26	ND
11	C20	7.26±1.40	2.45±1.49	1.61±0.40	0.56±0.10
12	3-MeC20	ND	ND	0.50±0.16	ND
13	C21	5.16±1.30	2.60±1.55	1.11±0.23	0.61±0.11
14	5-MeC21	ND	ND	0.53±0.14	ND
15	3-MeC21	ND	ND	0.52±0.14	ND
16	Docosene*	ND	ND	0.70±0.17	ND
17	C22	10.06±2.06	4.68±2.47	2.15±0.34	1.12±0.17
18	C23	10.40±1.94	6.54±3.30	2.06±0.23	1.63±0.40
19	C24	9.38±1.83	8.26±3.82	1.74±0.22	2.26±0.88
20	C25	6.77±1.17	9.67±3.62	2.20±0.61	2.95±1.31
21	4-MeC25	ND	ND	ND	0.50±0.11
22	3-MeC25	ND	ND	ND	0.50±0.08
23	C26	3.11±0.75	9.94±4.07	0.84±0.15	3.33±1.70
24	3-MeC26	ND	ND	ND	0.50±0.12
25	C27	2.52±0.65	11.81±2.97	9.81±2.70	5.66±1.98
26	3-MeC27	ND	1.18±0.82	ND	0.51±0.16
27	C28	6.82±1.36	11.57±3.17	3.26±0.60	4.09±1.70
28	3-MeC28	ND	1.26±0.85	ND	0.50±0.14
29	C29	13.23±4.82	10.69±3.28	39.67±4.37	32.05±7.38
30	7-MeC29	ND	ND	ND	0.55±0.14
31	4-MeC29	ND	ND	ND	0.78±0.37
32	3-MeC29	ND	ND	ND	0.57±0.17
33	C30	ND	9.17±4.04	3.43±0.41	4.78±1.49
34	5-MeC30	ND	ND	ND	1.03±0.57
35	4-MeC30	ND	ND	ND	0.56±0.22
36	3-MeC30	ND	ND	ND	0.99±0.61

Peak No.	Peak ID	<i>L. sericata</i> (UK) first day	<i>L. sericata</i> (DE) first day	<i>L. sericata</i> (UK) last day	<i>L. sericata</i> (DE) last day
37	C31	ND	8.80±4.05	18.53±1.79	25.34±4.76
38	13+15-MeC31	ND	ND	ND	0.68±0.24
39	11+13-MeC32	ND	ND	ND	0.52±0.19
40	3-MeC31	ND	ND	ND	0.59±0.17
41	C32	ND	ND	0.97±0.27	2.74±1.14
42	C33	ND	ND	2.32±0.42	4.93±1.37

ND Not detected

* Double bond positions were not determined

When the compounds detected in the chemical profiles of the first instar larvae were analysed according to the CHC classification (Table 6.9), a total of 20 CHCs were detected in the UK population. A total of 15 CHCs were detected in the German population. The numbers of n-alkanes, alkenes and branched methyl alkanes detected in the profiles were nearly identical. Similar numbers but different concentrations of n-alkanes were detected in both populations. There was no common branched methyl alkane detected in both profiles. Branched methyl alkanes of C17:H to C19:H and their isomers were detected only in the UK population. The 3-MeC27 and 3-MeC28 branched methyl alkanes were detected only in the German population. Eicosene was the only alkene detected only in the UK population, with a concentration of 3%. Other alkenes were not included in the assessment due to their lower concentrations.

Table 6.9: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *L. sericata* first instar larvae reared outdoors in UK and Germany

	<i>L. sericata</i> (UK) first day		<i>L. sericata</i> (DE) first day	
	number	Total % peak area	number	Total % peak area
n-alkanes	14	87	13	98
Branched methyl HC	5	9	2	2
Alkenes	1	4	-	-
Total	20	100	15	100

The GC chromatograms of the first instar larvae extracted from the UK and German populations are given in Figure 6.11. When the chromatograms were examined, it was seen that CHCs with shorter carbon chain lengths were found in higher concentrations in the UK population. Conversely, CHC with longer carbon chains were higher concentrations in the German population.

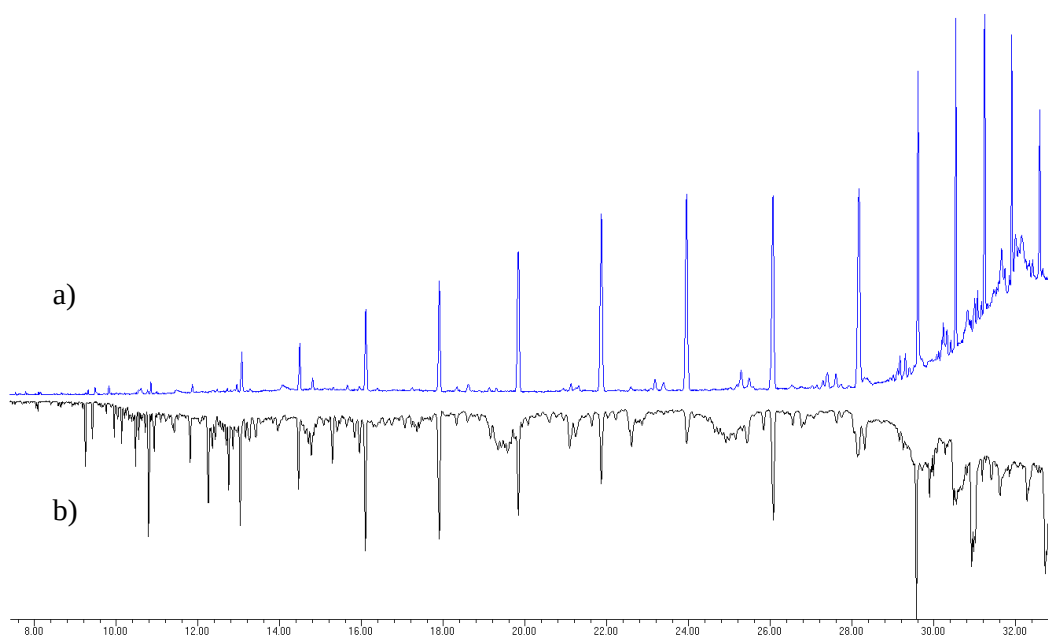


Figure 6.11: GC chromatograms showing CHC profiles of *L. sericata* first instar larvae reared in a) Germany, b) the UK

When the percentage concentrations of n-alkenes were examined in detail in Figure 6.12, it was found that n-alkane concentrations from C16:H to C24:H were higher in the UK population. At the same time, the longer chain length CHCs were abundant in the German population. C29:H n-alkane was the predominant compound in the UK population, and its concentration was higher than in the German population. The predominant compound in the German population was C27:H n-alkane. The total percentage concentrations of n-alkanes detected in the German population were 11% higher than in the UK. On the other hand, the UK population contained 7% more branched methyl alkanes in total concentration than the German population. Eicosene was the only alkene detected in the UK population, accounting for 4% of the total CHC concentration.

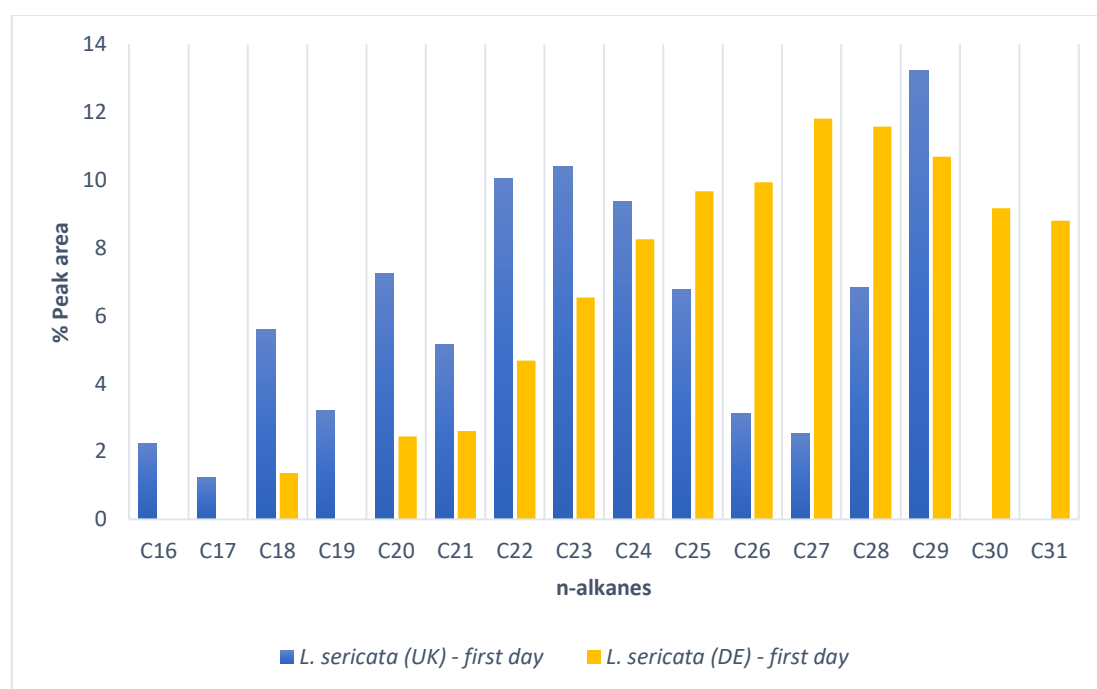


Figure 6.12: Plot showing the percentage concentrations of n-alkanes detected in *L. sericata* first instar larvae reared outdoors in the UK and Germany

L. sericata larvae were reared in cages placed in fields in the UK and Germany by exposure to natural ambient temperature, just like *C. vicina* larvae. An individual datalogger recorded the ambient temperature at hour intervals. The mentioned temperature data are given in Appendices 1 and 7. The larvae reared in the UK were exposed to the average temperature of 19.9°C on the first day, and the larvae reared in Germany were exposed to the average temperature of 19.0°C. Comparative temperature data covering the larval period is also given in Appendix 31. When comparing the temperature data, it was found that the first instar larvae reared in the UK were exposed to higher temperatures during Day 1 than the first instar larvae reared in Germany. Moreover, *L. sericata* blowfly eggs were exposed to cooler temperatures for almost the first 24 hours until larvae hatched from the eggs. On the contrary, blowfly eggs reared in Germany were exposed to higher temperatures during the egg stage.

Figure 6.13 shows GC chromatograms of post-feeding larvae of *L. sericata*, reared outdoors in the UK and Germany, extracted on the last larval day just before the puparial stage. CHC profiles of *L. sericata* larvae on the last day of the larval period were quite similar, although there were differences between the peak areas of the compared larval populations. The predominant n-alkanes in the profiles of both populations were odd-numbered CHC chains containing from 27 to 31 carbon atoms. C29:H n-alkane was the most predominant in both populations, but the percentage concentration was higher in the UK. C27:H and C29:H were abundant in the UK population, while the C31:H n-alkane was more abundant in the German population.

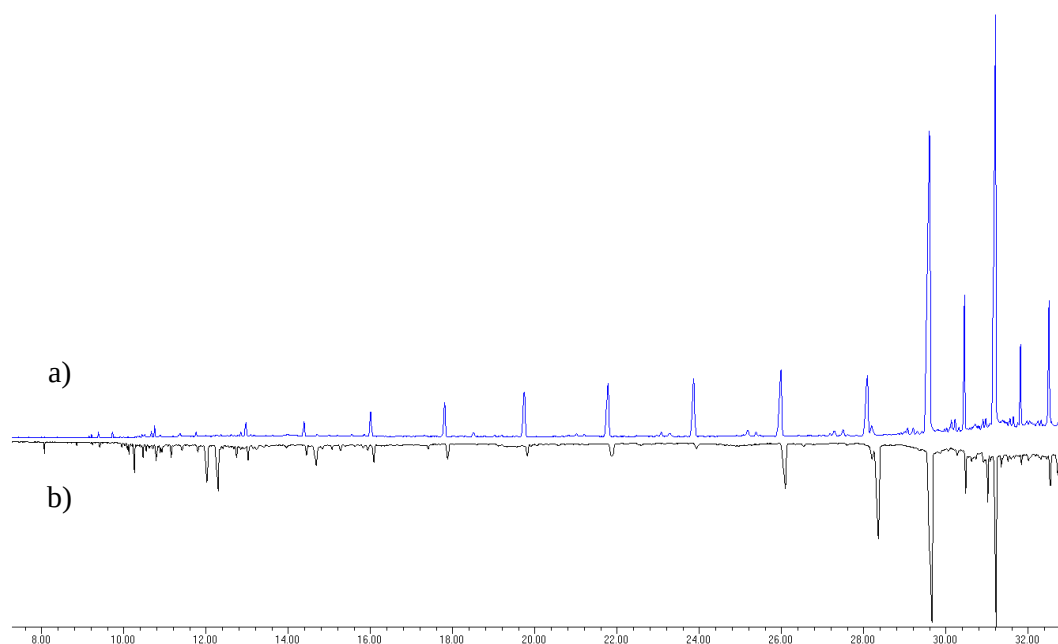


Figure 6.13: GC chromatograms showing CHC profiles of *L. sericata* post-feeding larvae reared in a) Germany, b) the UK

A total of 25 CHCs were detected in the UK population, and 30 CHCs were detected in the German (Table 6.10). All n-alkanes were detected as common in both populations. However, there were differences in percentage concentrations: 7 branched methyl alkanes were detected in the UK population and 14 in German. No common branched methyl alkane was detected in the populations. Branched methyl alkanes with carbon numbers C17:H and C21:H and their isomers were detected in the UK population, while branched methyl alkanes with carbon numbers C25:H and C31:H and their isomers were detected in the German population. Although more branched methyl alkanes were detected in the German population, the total percentage concentrations of these compounds in the chemical profiles were the same. Eicosene and docosene were alkenes detected only in the UK population, accounting for 2% of the total CHC concentration. Since the percentage concentration of the other alkenes detected was below 0.5%, they were omitted.

Table 6.10: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *L. sericata* post-feeding larvae reared outdoors in UK and Germany

	<i>L. sericata</i> (UK) last day		<i>L. sericata</i> (DE) last day	
	number	Total % peak area	number	Total % peak area
Alkanes	16	92	16	93
Branched methyl HC	7	6	14	7
Alkenes	2	2	-	-
Total	25	100	30	100

The data given in Table 6.8 were applied to PCA to visualise the differences detected and differentiate the first instar larvae, and post-feeding larvae populations of *L. sericata* reared in the UK and Germany. PCA was performed using six principal components, describing 98.1% of the dataset variation, with the first three principal components being 80.7, 12.8 and 4.6%, respectively. The PCA plot in Figure 6.14 was created using the PC2, PC3 and PC4 components, where the separation between the clusters was apparent.

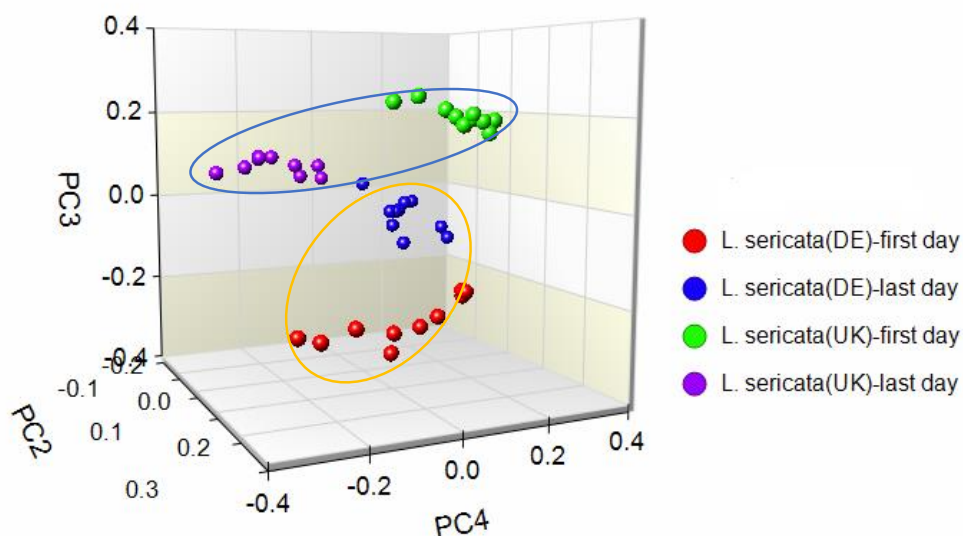


Figure 6.14: PCA plot showing PC3 vs PC2 vs PC4 for *L. sericata* larvae reared outdoors in the UK and Germany, using n-alkanes, alkenes and methyl branched alkanes, clusters were grouped by populations

Discriminant analysis was performed to determine which CHCs were most responsible for separating populations of UK and German *L. sericata* first instar and post-feeding larvae. Among all the CHCs, given in Table 6.8, the program identified tetracosane (C24:H), nonacosane (C29:H), triacontane (C30:H) and hentriacontane (C31:H) as the most important CHCs in the discriminant analysis.

On the plot created according to the PCA data, the UK and German populations of *L. sericata* were clearly separated from each other on both the first and last days of the larval

stage. The tight distribution of the repeat samples forming the clusters in the plot also showed that the variation and reproducibility of the results were high. Considering the similarity of the chemical profiles of the post-feeding larvae on the last day of the larval period, being able to achieve this level of discrimination demonstrates the success of the CHC analysis and PCA analysis. There was no overlap between populations. Despite the effects of temperature and different populations on CHC profiles, differentiation was successfully made between the populations of the same species.

6.3.2 Discussion of Larval Stage Comparison

Differences in CHC composition between populations are affected by environmental factors, revealing habitat diversity. Temperature is one of the most critical environmental factors affecting the cuticle structure of populations. Toolson and Hadley [175] found that populations living in warmer climates had a higher concentration of long-chain n-alkanes and showed that environmental factors affect the CHC profile. This study's results demonstrated the effect of temperature on populations' CHC profiles, with hot and dry conditions leading to decreased shorter chain compound concentrations that readily volatilise.

Catts [172] stated that ambient weather temperatures play a predominant role in developing eggs and immature larvae, but this decreases rapidly as the corpse cools, and immature larvae develop simultaneously with fluctuations in ambient temperature. In this study, the interpretation drawn from the results is that the temperature to which the blowfly eggs are exposed plays a significant role in shaping the CHC profiles of the first instar larvae. While CHCs with shorter carbon chains were in the majority in the CHC profiles of the UK population exposed to lower temperatures in the egg stage, CHCs with longer carbon chains were predominant in the CHC profiles of the German population because they were exposed to higher temperatures. The temperature during the egg stage was found to have a major impact on the formation of CHC profiles in both *C. vicina* and *L. sericata* first instar larvae.

Greenberg [52] compared the ADH of *L. sericata* in the Midwest USA with data obtained by Marchenko [176] in Russia using the same species. Midwest larvae reached the adult stage with 7684 ADH, while Russian adult flies reached 8395 ADH. When ADH values

were calculated in the current study using the temperature data recorded during the larval period, it was found that the UK larvae reached the adult stage with 9977 and German larvae reached the adult stage with an accumulated 12684. The current comparison between the same species living in different habitats also revealed the disadvantage of using the ADD/H method for ageing. Obtaining different values for the same species in different populations makes the use of standard key data for the ADD/H method questionable. It also showed the effect of temperature on the ADD/H method for a species growing under fluctuating temperatures.

The cuticle layer is formed to protect against water loss when the creature is exposed to higher temperatures. Toolson and Kuper-Simbron [98] stated that CHC compositions might be highly variable and sensitive to environmental influences. They found that *Drosophila pseudoobscura* can rapidly change their profiles in laboratory conditions. The results showed that prolonged maintenance in laboratory conditions led to significant changes in CHC profiles. Howard et al. [177] also supported the claim that insects respond relatively quickly to environmental challenges. When the temperature data were examined, it was understood that the reason for the percentage concentration of longer carbon chain CHCs in the chemical profiles of the first instar and post-feeding stage *L. sericata* and *C. vicina* larvae reared in Germany might be the maximum temperature they were exposed to. This situation can be shown as evidence of the effect of temperature as the CHC profiles of the species is being formed. Also, these results strongly imply the importance of the CHC profile in the water loss process.

In the research, to compare the population, the extractions obtained on the first day of the larval stage and the last days just before the puparial stage were taken as the basis. The main factor affecting the development rate and duration of the larval period within the same species among different geographical regions is temperature. Discriminant analysis of the CHC profiles separated the larvae by both location and age. These results support that it has potential forensic applications in determining corpse relocation and examining population ecology. The difference in the CHC profiles of the larvae may provide evidence of when relocation of a corpse occurred. Differences in environmental conditions between the UK and Germany, where the larvae were reared, explain the differences in the CHC composition of the species. Results showing CHC constituents

and concentrations revealed that a species is associated with cuticle permeability and transition temperature.

The longest n-alkanes and branched methyl alkanes that a given species can synthesise are deposited to achieve lower cuticle permeabilities. Hadley [168] showed that cuticle permeability decreases when the rate of water loss increases. In contrast, it was observed that increasing the percentage of branched methyl alkanes increases the cuticle permeability, as the methyl branches prevent the CHC chains from being closely packed. When the CHC compositions in the larval profiles and the temperature data were examined, the increase in the number of CHCs with longer carbon chains in the chemical profiles was compatible with findings reported in the literature. The results obtained in this section of the chapter also support these claims. Both *C. vicina* and *L. sericata* larvae responded quite rapidly to temperature changes.

In order to examine different populations of the same species independently of temperature, studies with species collected from different regions reared under constant temperature will only reveal genetic differences in different populations. Therefore, future studies conducted in this context can identify genetic traits.

6.4 Puparial Stage

Forensic entomologists use published data on insects collected from crime scenes to estimate the PMI_{min}. However, these data only represent populations of insects collected from specific locations. Studies suggest that populations may exhibit genetic variation in their development according to environmental differences [178,179]. CHC composition is used as an essential taxonomic character, as insects biosynthesise most of their CHC layer. The cuticles of the larvae are less sclerotised (i.e., less solid), so they are more susceptible to dehydration than in other life stages. However, the larvae feed on a relatively moist substrate that provides them protection.

The pupae stage is an immobile stage, and protection against dehydration is vital; when the larvae face threats such as sunlight or lower relative humidity, they can move away, but pupae cannot escape exposure [85]. Therefore, a cuticle that provides effective waterproofing to the larvae may avoid fatal impacts. A cuticle is indispensable for the

puparial stage. Goodrich [127] examined the cuticle compounds of pupae and adult flies of *Lucilia cuprina* and found qualitative and quantitative differences between these stages. Likewise, Owings et al. [180] showed significant differences in puparial development time and size between *Cochliomyia macellaria* derived from populations in distinct geographical locations and years. Therefore, CHC analysis can be used as a valuable tool for forensic investigations.

This study aimed to determine the inter-population variation of the pupae of *C. vicina* and *L. sericata* species reared in different habitats based on qualitative and quantitative changes in CHC profiles extracted from the epicuticle and the ratio of n-alkanes to branched alkanes. Since CHC composition can be changed with the developmental stage and temperature, the extractions included different age periods at the beginning and end of the developmental stage of pupae and by recording the temperature data and examining the temperature effect.

6.4.1 Results for Puparial Stage

6.4.1.1 *C. vicina*

The CHC profiles of *C. vicina* pupae were extracted on the first pupae day and the last pupae day before the emergence of adult flies reared outdoors in the UK and Germany. The CHC profiles were identified by GC-MS analysis; CHC compounds are given in Table 6.11. The chain lengths of the detected CHCs ranged from C16:H to C33:H. The data obtained were used for the subsequent PCA analysis. CHC compounds with concentrations below 0.5% were ignored.

Table 6.11: Compounds extracted from the first day and last day of the pupae stages *C. vicina* reared outdoors in the UK and Germany, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>C. vicina</i> (UK) first day	<i>C. vicina</i> (DE) first day	<i>C. vicina</i> (UK) last day	<i>C. vicina</i> (DE) last day
1	C16	1.79±0.41	ND	1.02±0.22	ND
2	C17	ND	ND	0.51±0.12	ND
3	3-MeC17	1.40±0.18	ND	0.99±0.13	ND
4	Octadecene*	0.57±0.10	ND	0.55±0.07	ND
5	C18	3.07±0.37	0.79±0.17	2.96±0.33	ND
6	3-MeC18	1.60±0.18	ND	1.30±0.27	ND
7	3-MeC19	ND	0.80±0.17	ND	ND
8	Eicosene*	ND	ND	0.55±0.21	ND
9	C20	2.62±0.30	1.56±0.24	2.71±0.72	ND
10	3-MeC20	1.72±0.28	ND	1.87±0.75	ND
11	3-MeC21	ND	1.47±0.65	ND	ND
12	Docosene*	0.81±0.12	ND	2.55±0.70	ND
13	C22	1.94±0.24	1.53±0.48	2.97±1.05	ND
14	C23	ND	0.79±0.37	0.55±0.42	ND
15	3-MeC23	1.14±0.14	0.79±0.12	1.33±0.30	ND
16	C24	1.22±0.15	0.92±0.14	1.39±0.36	ND
17	C25	49.95±8.94	55.12±6.38	33.89±7.35	64.93±15.63
18	11+13-MeC25	ND	0.70±0.09	0.55±0.16	ND
19	3-MeC25	1.08±0.16	ND	1.44±0.30	ND
20	C26	2.43±1.09	1.41±0.28	2.06±0.50	1.06±0.45
21	C27	10.22±2.52	12.68±4.27	15.05±4.72	12.60±7.63
22	11+13-MeC27	ND	ND	0.89±0.20	ND
23	3-MeC27	1.59±0.39	1.58±0.48	2.69±0.72	1.30±0.51
24	C28	1.43±0.26	2.10±0.47	1.74±0.42	2.30±1.04
25	4-MeC28	ND	ND	0.54±0.15	ND
26	3-MeC28	ND	ND	0.87±0.25	ND
27	C29	6.31±1.31	9.28±1.73	7.93±1.75	9.64±4.56
28	3-MeC29	0.67±0.18	0.71±0.18	0.85±0.16	0.68±0.41
29	C30	0.61±0.24	1.13±0.42	0.63±0.15	1.35±0.39
30	8-MeC30	ND	ND	0.56±0.27	ND
31	4-MeC30	ND	ND	0.58±0.11	ND
32	C31	2.83±0.75	2.49±0.42	2.81±0.62	2.40±0.60
33	13+15-MeC31	1.63±0.45	ND	1.34±0.42	ND
34	3-MeC31	1.11±0.38	1.24±0.49	0.71±0.20	ND
35	C32	1.17±0.44	1.45±0.48	1.33±0.36	2.17±0.51
36	C33	1.09±0.44	1.44±0.14	1.37±0.63	1.57±0.29

ND Not detected

* Double bond positions were not determined

The CHCs detected in the first-day samples of *C. vicina* pupae are given in Table 6.12. A total of 25 CHCs were detected in the CHC profiles of pupae extracted from the UK population and 22 CHCs in the German population. The exact number of n-alkanes and close numbers of branched methyl alkane compounds were detected in both populations. However, two alkene compounds with total concentrations of 1% were detected only in the UK population. Alkenes with concentrations less than 0.5% were not evaluated.

Table 6.12: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *C. vicina* the first-day pupae reared outdoors in the UK and Germany

	<i>C. vicina</i> (UK) first day		<i>C. vicina</i> (DE) first day	
	number	Total % peak area	number	Total % peak area
n-alkanes	14	87	14	93
Branched methyl HC	9	12	8	7
Alkenes	2	1	-	-
Total	25	100	22	100

GC chromatograms showing the chemical profile of first-day pupae of the UK and German populations are given in Figure 6.15. The chromatograms were quite similar except for the peak areas of the compounds. In both the UK and the German populations, n-alkanes consisted of 90% of the chemical profiles, with the most abundant n-alkanes being C25:H, C27:H and C29:H. However, the percentage concentration of both these n-alkanes and the total n-alkanes was 6% higher in the German population.

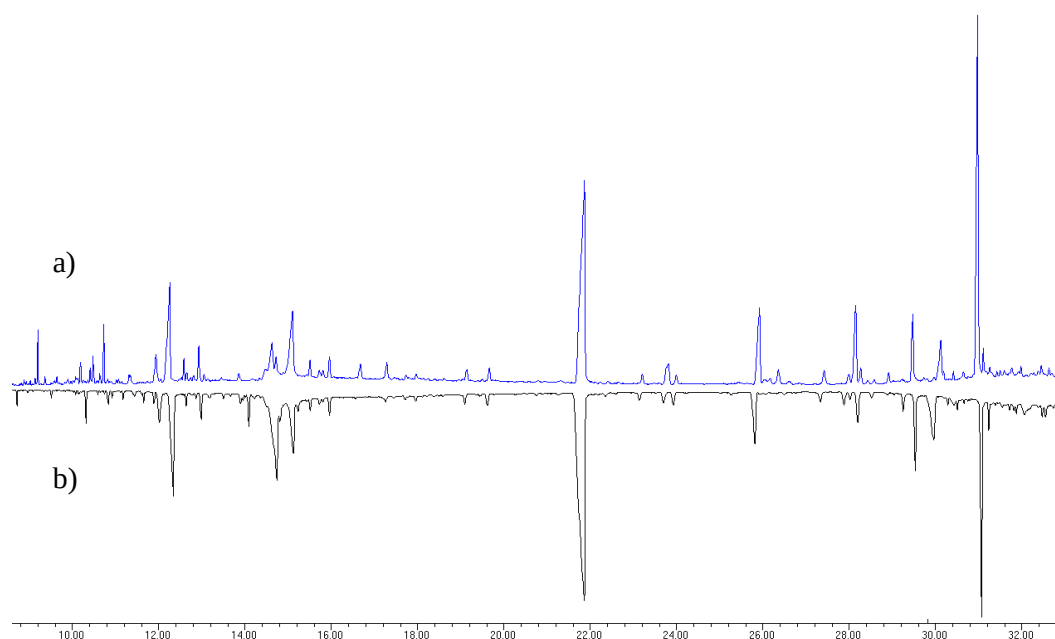


Figure 6.15: GC chromatograms showing the first day of pupae stages of *C. vicina* species reared in a) the UK, b) Germany

Although the percentage concentrations of branched methyl alkanes were deficient, they were necessary for distinguishing between populations. Appendix 32 indicates that 3-MeC23, 3-MeC27, 3-MeC29 and 3-MeC31 were detected in both populations and almost similar percentage concentrations. On the other hand, 3-MeC19, 3-MeC21 and 11+13-MeC25 were only detected in the UK population. Other branched methyl alkanes given in the table were detected only in the German population. The percentage concentration of total branched methyl alkane compounds detected was 5% higher in the UK population.

The chemical profiles extracted on the first and last days of the pupae stage was quite similar. The visual difference between the GC chromatograms of the populations was that the peak areas of the compounds detected in the German population were higher than in the UK (Figure 6.16).

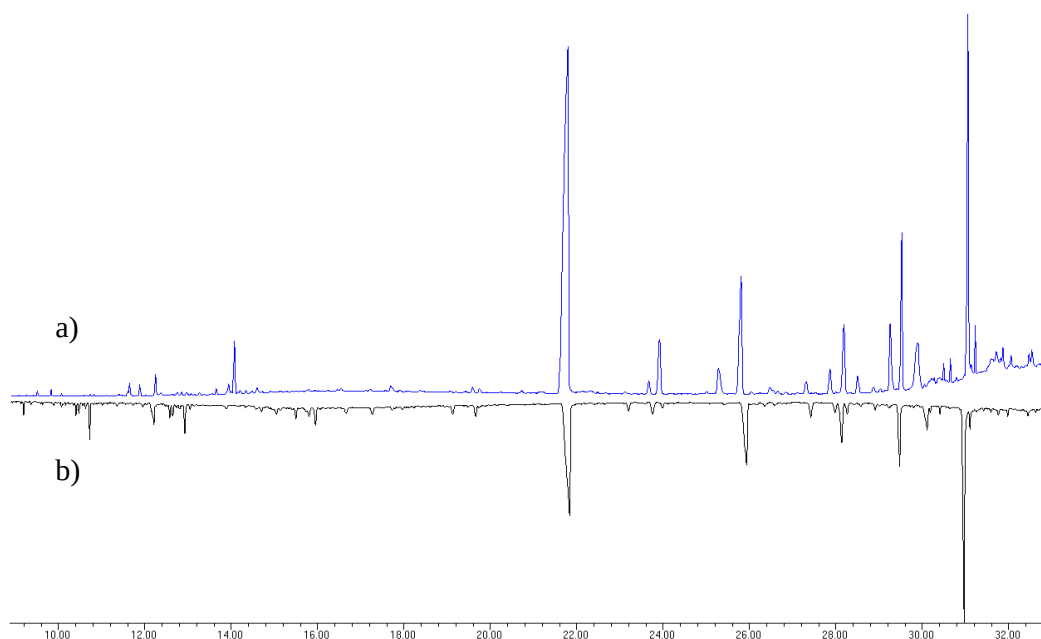


Figure 6.16: GC chromatograms showing the last day of pupae stages of *C. vicina* species reared in a) Germany, b) the UK

A total of 34 CHC compounds were detected in the UK population and 11 CHCs in the German population (Table 6.13). CHC compounds with carbon lengths C16:H and C33:H were detected in the UK population, although their concentration was relatively lower (>0.5%). In the German population, CHCs with carbon numbers of C25:H and C:33H were detected. The number of n-alkanes detected in the UK population was twice, and the number of branched methyl alkanes was nine times higher than those of the German population. Only 3-MeC27 and 3-MeC29 detected in the UK population were also detected in the German population. Like in the first day samples, the most abundant compounds in the last day samples were C25:H, C27:H and C29:H. The percentage concentration of the C25:H n-alkane in the German population was about twice the percentage concentration in the UK; in contrast, the percentage concentrations of the other two n-alkanes in the populations were similar. Octadecene, docosene and eicosene alkenes were only detected in the UK population, accounting for 4% of the total CHC concentration. They were omitted since the percentage concentration of alkenes detected in the German population was below 0.5%.

Table 6.13: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *C. vicina* on the last day pupae reared outdoors in UK and Germany

	<i>C. vicina</i> (UK) last day		<i>C. vicina</i> (DE) last day	
	number	Total % peak area	number	Total % peak area
n-alkanes	16	79	9	98
Branched methyl HC	15	18	2	2
Alkenes	3	4	-	-
Total	34	100	11	100

Although the chromatograms of the UK and German populations were similar in general appearance, the detection of CHCs with shorter carbon chains even in lower concentrations ($>0.5\%$) in the UK population was remarkable in terms of distinguishing between populations. For this reason, the temperatures to which pupae reared in the UK and Germany were exposed are given in Appendix 33 to comparatively examine the temperature effect on puparial CHC profiles. Appendix 33 shows that the German population was exposed to higher temperatures than the UK population. The temperature effect on the chemical profiles of the pupae can explain the higher total n-alkane concentration detected in the German population on the first day of puparia. Pupae reared in Germany were exposed to higher temperatures during the puparial stage, while those reared in the UK were exposed to a cooler environment. The presence of shorter carbon chain CHCs detected in the CHC profile of the UK population supports the effect of temperature on the CHC profile of the puparia.

The data given in Table 6.11 were applied to PCA to distinguish between the populations of the *C. vicina* pupae reared outdoors in the UK and Germany on the first and last days of the puparial stage and visualised the results obtained. PCA was performed using six principal components, describing 99.9% of the dataset variation, with the first three principal components being 98.4, 1.2 and 0.3%, respectively. 3D plots were used to clearly visualise the separation between clusters. In order to create the PCA plot given in Figure 6.17, PC1, PC2 and PC3 scores were preferred, where the separation between the clusters was more clearly achieved. It was aimed to evaluate the effects of geographical differences on CHC profiles using PCA to examine inter-population CHC profile variation.

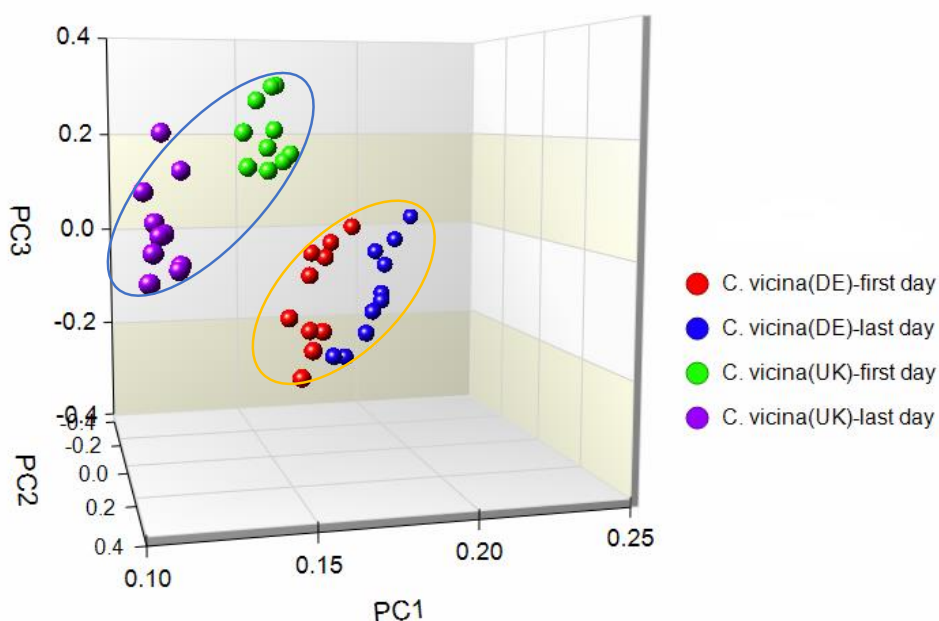


Figure 6.17: PCA plot showing PC3 vs PC2 vs PC1 for *C. vicina* pupae reared outdoors in the UK and Germany, using n-alkanes, alkenes and methyl branched alkanes, populations grouped clusters

Discriminant analysis was performed to determine which CHCs were most responsible for separating UK and German *C. vicina* populations on the first and last days of the puparial stage. Among all the CHCs (Table 6.12), the program identified octadecane (C18:H), pentacosane (C25:H), heptacosane (C27:H) and nonacosane (C29:H) as the most important CHCs in the discriminant analysis.

The ageing of *C. vicina* pupae was detailed in Chapter 5; here, this is pertinent with regard to distinguishing between age and population differences. The PCA plot shows that the differentiation according to age and population was successfully performed with the CHC analysis in the puparial stage, where even species identification and ageing are difficult with conventional morphological methods. The tight distribution of the repeat samples forming the clusters on the plot also showed that the variation and reproducibility of the results were high. Although clusters of the German population were located close together, there was no overlap between populations.

6.4.1.2 *L. sericata*

The CHCs on the chemical profiles of pupae were analysed by GC-MS. The CHC compounds identified in the CHC profiles of pupae on the first day and last day of the puparial period reared outdoors in the UK and Germany are given in Table 6.16. The chain lengths of the detected CHCs range from C20:H to C33:H. The data obtained were used for the subsequent PCA analysis. CHC compounds, especially alkenes with concentrations below 0.5%, were ignored for comparison.

Table 6.14: Compounds extracted from the first day and last day of the puparial stages *L. sericata* reared outdoors in the UK and Germany, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>L. sericata</i> (UK) first day	<i>L. sericata</i> (DE) first day	<i>L. sericata</i> (UK) last day	<i>L. sericata</i> (DE) last day
1	Eicosene*	ND	ND	0.66±0.06	ND
2	C20	ND	ND	0.59±0.08	ND
3	Docosene*	ND	ND	0.59±0.03	ND
4	C25	7.38±1.50	18.66±6.99	5.50±0.60	2.97±1.41
5	C26	ND	0.55±0.11	0.81±0.30	0.69±0.41
6	C27	20.20±4.85	12.81±3.81	23.24±6.74	12.92±6.28
7	3-MeC27	ND	0.74±0.32	1.41±0.71	2.96±2.04
8	C28	3.86±0.70	2.58±0.79	3.77±0.81	2.15±0.50
9	C29	48.92±8.48	41.19±13.22	46.01±8.29	32.43±5.45
10	13+15-MeC29	ND	0.70±0.25	ND	ND
11	11+13-MeC29	ND	0.50±0.11	ND	3.53±1.92
12	7-Mec29	0.58±0.23	0.53±0.94	0.57±0.20	ND
13	3-MeC29	ND	2.79±1.10	ND	6.06±3.45
14	C30	2.36±0.49	2.21±0.72	2.25±0.52	2.52±0.66
15	8-MeC30	ND	ND	ND	1.50±0.61
16	C31	14.11±3.18	14.22±4.73	11.94±2.21	29.13±47.40
17	11+13-MeC31	ND	1.01±0.34	ND	ND
18	C32	1.46±0.40	ND	1.37±0.38	1.56±0.61
19	C33	1.11±0.25	1.72±0.55	0.97±0.24	1.58±0.30

ND Not detected

* Double bond positions were not determine

When the CHCs detected in the first-day pupae were classified (Table 6.15), nine CHC compounds were detected in the UK population and 14 in the German population. In both populations, the chemical profiles consisted mainly of n-alkanes. A total of eight n-alkanes were detected in both populations. In addition to 7-MeC29, the only branched methyl alkane detected in the UK population, six branched methyl alkanes were detected in the German population. Since the percentage concentrations of alkene compounds in both populations were below 0.5, they were not evaluated.

Table 6.15: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *L. sericata*, the first-day pupae reared outdoors in UK and Germany

	<i>L. sericata</i> (UK) first day		<i>L. sericata</i> (DE) first day	
	number	Total % peak area	number	Total % peak area
n-alkanes	8	99	8	94
Branched methyl HC	1	1	6	6
Alkenes	-	-	-	-
Total	9	100	14	100

The GC chromatograms of the first-day pupae (Figure 6.18) were quite similar, except for the difference in peak areas of the predominant compounds between populations. Of the most concentrated C25:H, C27:H, C29:H and C31:H n-alkanes, C27:H and C29:H were higher in the UK, while C25:H was higher in the German population.

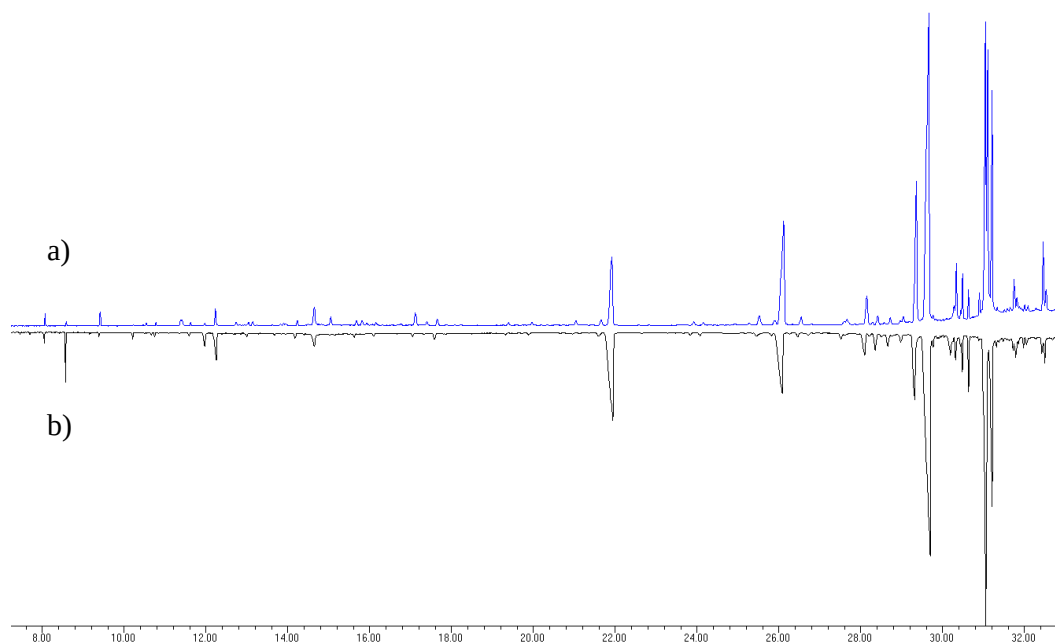


Figure 6.18: GC chromatograms showing the first day of pupae stages of *L. sericata* species reared in a) the UK, b) Germany

Apart from the percentage concentrations of n-alkanes that were predominant in the populations, the chemical profiles of the pupae on the last day of the puparial stage were also quite similar (Figure 6.19). The peak areas of the C25:H, C27:H, C29:H and C31:H n-alkanes made the apparent main difference in the GC chromatograms. C25:H, C27:H, and C29:H n-alkanes were more prevalent in the UK population, while the C31:H n-alkane was more notable in the German population.

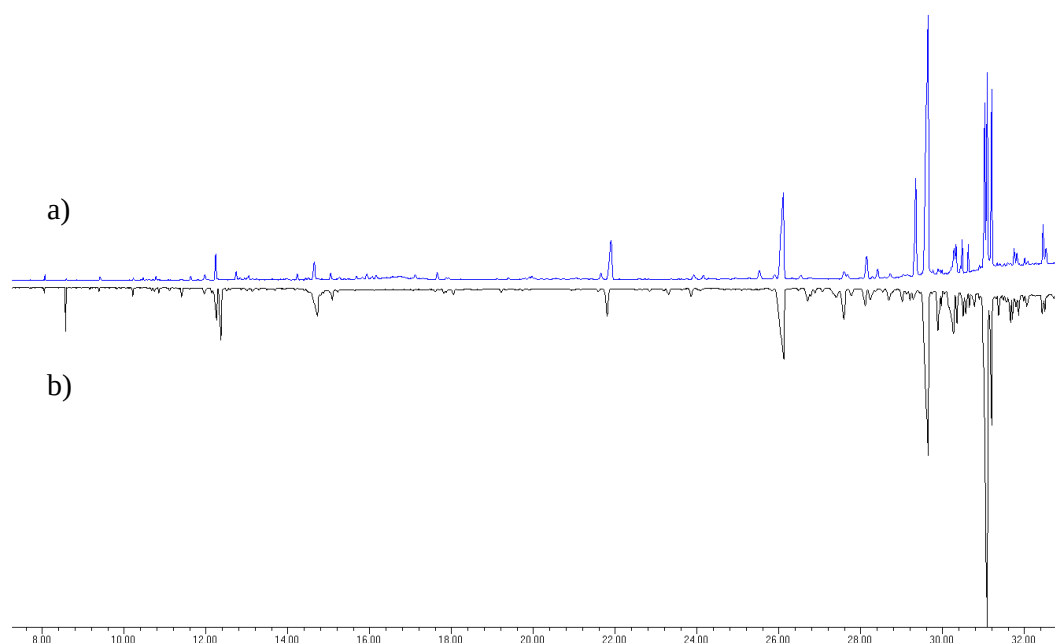


Figure 6.19: GC chromatograms showing the last day of pupae stages of *L. sericata* species reared in a) the UK, b) Germany

The compounds detected in the last day pupae of *L. sericata* are given in Table 6.16 according to the CHC classification. A total of 14 CHCs were detected in the CHC profiles of pupae extracted from the UK population and 13 CHCs in the German population. The only branched methyl alkane detected in both populations was 3-MeC27, and its percentage concentration was higher in the German population. Thus, the number and concentration of branched alkanes detected in the German population were higher than in the UK. On the other hand, alkene compounds were not detected in sufficient concentration in the German population, while eicosene and docosene were detected in 1% concentration in the UK population.

Table 6.16: CHC composition – numbers and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *L. sericata*, the last-day pupae reared outdoors in UK and Germany

	<i>L. sericata</i> (UK) last day		<i>L. sericata</i> (DE) last day	
	number	Total % peak area	number	Total % peak area
Alkanes	10	97	9	86
Branched methyl HC	2	2	4	14
Alkenes	2	1	-	-
Total	14	100	13	100

The temperatures in which *L. sericata* pupae were reared outdoors in the UK and Germany during the puparial period are given in Appendix 34. The puparial period lasted ten and 14 days in the UK and Germany, respectively. Branched methyl alkanes were also detected in the German population, exposed to a more fluctuating temperature.

Although the chemical profiles for pupae from the populations of the UK and Germany were similar, there were population-specific differences in the proportions of the CHC profiles extracted. The identified CHCs in the chromatograms were applied to PCA to separate the populations of the *L. sericata* pupae reared outdoors in the UK and Germany on the first and last days of the puparial stage to visualise the results obtained. PCA was performed using six principal components, describing 98.7% of the dataset variation, with

the first three principal components being 91.4, 5.2 and 2.0%, respectively. 3D plots were used to clearly visualise the separation between clusters. In order to create the PCA plot given in Figure 6.20, PC1, PC2 and PC3 scores were preferred, where the separation between the clusters was more clearly achieved. It was aimed to evaluate the effects of geographical differences on the CHC profile to examine the CHC profile variation between populations.

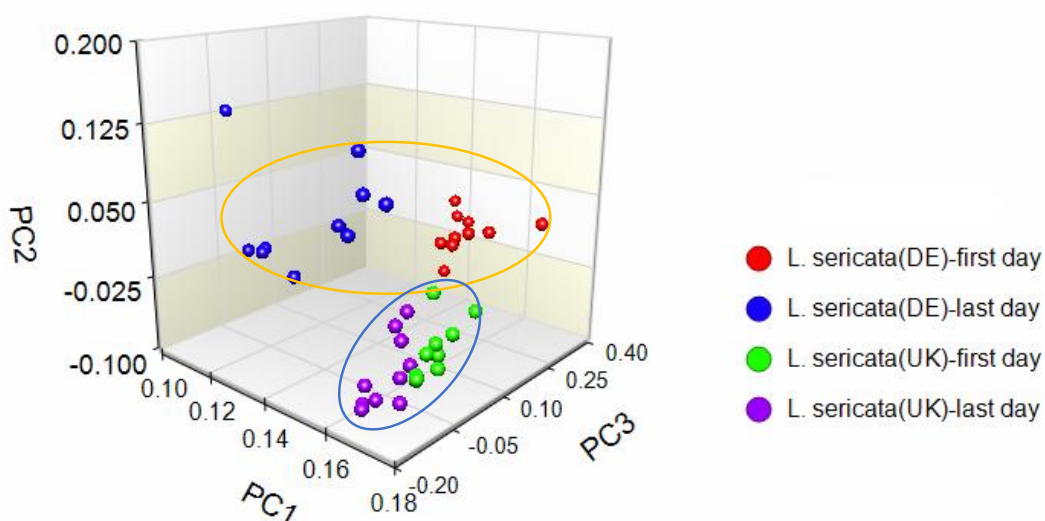


Figure 6.20: PCA plot showing PC2 vs PC1 vs PC3 for *L. sericata* pupae reared outdoors in the UK and Germany, using n-alkanes, alkenes, and methyl branched alkanes, populations grouped clusters

Discriminant analysis was performed to determine which CHCs were most responsible for separating UK and German *L. sericata* populations on the first and last days of the puparial stage. Among all the CHCs (Table 6.16), the program identified pentacosane (C25:H), heptacosane (C27:H), nonacosane (C29:H) and hentriacontane (C31:H) as the most important CHCs in the discriminant analysis.

The other clusters showed a tight distribution on the PCA plot, except for the scattered cluster representing the last day pupae reared in Germany. The tight distribution indicates the higher variation and reproducibility of the results. While it is already a complicated

process to distinguish between two samples with similar chemical profiles of puparia, this research separated *L. sericata* pupae using CHC analysis according to both population and age. Although samples taken from the UK population were particularly clumped (located close together) in the plot, there was no overlap between populations. The results obtained using the CHC analysis and PCA showed detectable differences between populations at the pupae stage, where it is complicated to determine structural differences.

6.4.2 Discussion of Puparial Stage Comparison

The results of *L. sericata* and *C. vicina* pupae show great potential for a life stage currently ignored in taxonomic studies. The effect of temperatures on the CHC profile is evident in the puparial life stage as well as in the larval life stage. While more CHCs with short carbon chains were detected in pupae reared in cooler habitats, CHCs with longer carbon chains were detected in the structure of pupae reared in warmer habitats. Furthermore, the percentage concentration of long-chain n-alkanes in the CHC profiles of the pupae indicates that they form a more stable and protective layer than the short-chain CHCs.

In addition, the puparial stage has proven to be an unavoidable candidate for researching the effect of temperature on the cuticle and its active role in preventing water loss by changing the CHC profiles with temperature. The obtained similarities and differences in the CHC profile of pupae can be used to indicate the geographical origin of populations. It can be said that the detection of longer chain n-alkanes in the puparial stage compared to other life stages was higher since they live in drier environments and therefore require more cuticle impermeability. These results strongly imply the importance of the CHC layer in the water loss process. Branched methyl alkanes prevent tight molecular packing, which decreases membrane fluidity and permeability. The fact that organisms form a structure rich in n-alkanes and less in branched methyl alkane in the puparial stage, where they need protection most, indicates the importance of the protection the CHC profile provides for insects.

With the results presented, both the age estimation and population discrimination were made in the puparial stage. In addition, the effect of temperature on the pupae cuticle was shown to change with age.

6.5 Summary of Findings on Comparative Studies for the UK and German Populations

In most of the studies, species collected from the wild were reared under laboratory conditions. However, eggs obtained from adult female flies collected from the wild were reared in cages placed outdoors in this study and the temperature was recorded with an individual datalogger. In conventional forensic entomological applications, temperature data from the nearest weather station is used to estimate the larval development time. However, whether the conditions recorded at a meteorological station are valid even if there is a close recording station to the crime scene should be considered. Previous studies [7,150] have shown that weather station data and microhabitat data may differ. Although the general weather conditions are pretty similar, microhabitat conditions can differ significantly and may not be comparable; furthermore, vegetation, air drainage and slope exposure all significantly affect microhabitat conditions [172].

In order to determine differences between populations, detection or non-detection of compounds in the CHC profiles and their quantitative values were used. The same component can be detected in the two profiles; however, different ecological and physiological characteristics may cause differences in their quantitative composition. Therefore, qualitative and quantitative data make it easier to distinguish between populations. The results obtained in this section examined the effects of both population differences and temperature effects on CHC profiles. Future studies will explore species collected from different populations reared under constant temperatures, eliminating the temperature effect; thus, only population differences can be discussed.

Chapter 7

Comparative Studies of CHC Profiles of Adult Flies and Empty Puparia for the UK and German and Turkish Populations

7.1 Introduction

Epicuticular lipid composition has a general effect on the mechanisms of the transcuticular flow of water, as well as synthesis and deposition against desiccation and water loss. Furthermore, epicuticular lipid has an adaptive potential in inter-individual variance. The adaptive response of epicuticular lipid composition and consequent regulation of cuticular permeability fills a significant gap in understanding the general phenomenon of organisms adaptation to their environment [92]. It has been hypothesised that as insects adapt to new habitats or environments with different temperature and climate types, the composition of this waxy layer evolves to maintain water balance [76]. Existing literature mainly focuses on the epicuticular lipid composition while overlooking variations in its composition among individuals living in different habitats. Previously collected and lab-reared generations of insects were used for facilitating quantitative analysis, which further obscured individual variations.

The biophysical properties of surface epicuticular lipids consist mainly of long-chain saturated CHCs and other hydrophobic compounds. These structures exhibit differences between species, which is probably due in part to genetic differences and environmental factors [169]. Toolson and Hadley [175] examined the cuticular permeability and lipid composition of *Centruroides sculpturatus* in the summer and winter seasons and revealed

seasonal impacts on the cuticular structure and changes in the structure. Kruger and Pappas [179] demonstrated the geographical variation of the CHC profile among 14 populations of *Aedes albopictus*. Bontonou et al. [181] compared the CHC profile of *Drosophila melanogaster* from a natural population with those from highly inbred strains adapted to laboratory conditions and examined the role of CHCs in desiccation and sexual selection across all strains and lines. Chung and Carroll [76] investigated how ecological variation in insect CHCs could lead to differentiation in mating signals and reproductive isolation. These studies showed that the evolution of insect CHCs might be the best method for understanding ecological speciation.

Previous studies on beetles [168,170,177], wasps [97], cockroaches [121,122], grasshoppers [126,169], parasitoids [171,182], ants [91,119], mosquitoes [178,179,183,184] and fruit flies [76,167,181,185–188] have shown that insect populations of the same species found in separate geographical regions might have different CHC profiles. However, the number of studies on blowflies is very few [85,115,166].

Byrne et al. [166] determined the difference in *Phormia regina* blowfly species collected from three different geographical populations by CHC analysis with DA. In another study, Paula et al. [85] determined the chemotaxonomic profile and the intraspecific variation of CHC compounds in *Chrysomya megacephala* adult flies using GC-MS and discriminant function analysis. The results of both studies showed that there were qualitative and quantitative differences between populations and that longer carbon chain CHCs were more abundant in CHC profiles extracted from samples from warmer regions than from colder regions. Paula et al. [115] also performed a similar study using mid-infrared photoacoustic spectroscopy, and the results were similar. The different environmental conditions between the geographical regions were the reason for the differences in the CHC compositions: hot and dry environmental conditions lead to the loss of CHC with the short-carbon chain that easily volatilizes, and longer carbon chain CHCs provide more protection against desiccation.

In this study, CHCs of *L. sericata* and *C. vicina* species were extracted from individual adult flies. Their remaining empty puparia were collected from three geographical regions: Swindon (UK), Frankfurt (Germany) and Ankara (Turkey). Experiments were

conducted during the summer seasons of 2018 and 2019 to minimise the seasonal variation factor while detecting differences between populations. The distances between the populations were: over 740 km between the UK and Germany, over 2190 km between Germany and Turkey, and over 2930 km between the UK and Turkey (Figure 7.1). The extracts were subjected to GC-MS, and their CHC profiles were defined. The details of collecting adult flies were given in Section 3.2.

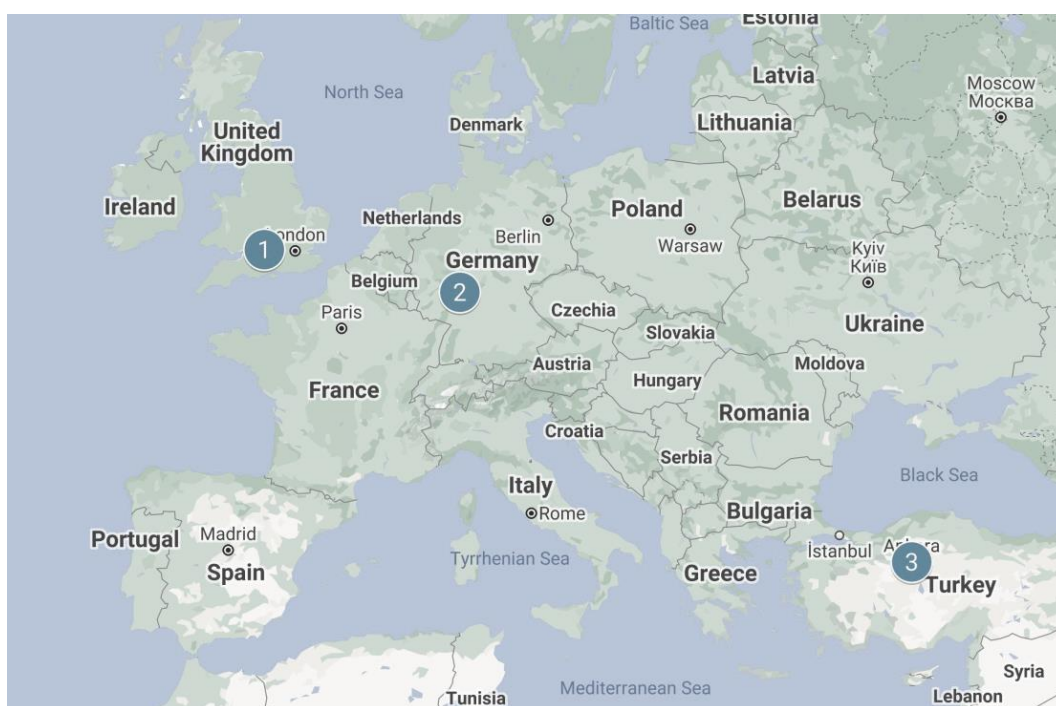


Figure 7.1: Locations of collection sites for *L. sericata* and *C. vicina* populations

7.2 Adult Fly Stage

Adult fly physiology is affected by some key factors that may be summarized briefly at the outset of this section. The epicuticle of insects is a protection shield against desiccation and intra- and interspecific interactions [189]. The compounds found in the cuticle have to maintain a balance between these two functions. Viscosity is associated with waterproofing, and fluidity is associated with effective communication. Temperature change causes the epicuticular surface to change to maintain body homeostasis [97]. Epicuticular surface lipids play a critical role in adapting arthropods to terrestrial environments by reducing the water loss through the cuticle. Differences in cuticle

composition are associated with water loss rates in inter- and intraspecific comparisons [190].

Prior research has shown that the cuticular chemical makeup of adult flies varies by population. Forensic investigators can utilise this evidence to establish where the crime happened and whether or not the corpse has been relocated [85,115]. Noorman and Den Otter [99] investigated the effects of relative humidity, temperature and population density on the CHC profiles of *Musca domestica* adult flies. Their results showed that the CHC profile was affected by external factors and the change was traceable. The general pattern is that populations living in warmer and drier habitats are expected to lose water more rapidly than populations living in cooler and moister habitats; also, longer chain-length hydrocarbons are expected to be abundant in their cuticle layers [87].

CHC profiles depend mostly on several factors, such as the age of the adult flies, rearing conditions and even the number of generations reared in the laboratory. Toolson et al. [151] compared the CHC compositions of laboratory-reared and wild-collected *Drosophila mojavensis* adult flies, demonstrating the effect of the rearing medium on the cuticle layer. Because the stocks reared in the laboratory are exposed to relatively more stable conditions, they lose most of their CHC surface lipids used against dehydration [98]. Therefore, in this study, samples collected from the wild were preferred since it was desired to examine the effect of environmental conditions such as temperature and population on the CHC profile of adult flies.

This study determined the variation between *C. vicina* and *L. sericata* species living in different habitats, the UK, Germany and Turkey, based on the qualitative and quantitative differences in CHC profiles extracted. First, CHC compounds were analysed by GC-MS. Then, by applying multivariate statistical analysis to the results, the distinction between species was obtained.

7.2.1 Results for Adult Fly Stage

7.2.1.1 *L. sericata*

The chemical profiles of *L. sericata* adult fly populations collected from the UK, Germany and Turkey were identified by GC-MS analysis to be a mixture of n-alkanes, alkenes and branched methyl alkanes. The CHCs compounds identified on the chromatograms are given in Table 7.1. The chain lengths of the detected CHCs range from C23:H to C33:H. CHCs with concentrations below 0.5% were not included for inter-population comparison. The positions of the double bonds in alkenes were not determined. Therefore, the same alkenes with a different location of the double bond were treated as different alkenes.

Table 7.1: Compounds extracted from adult flies of *L. sericata* collected from the UK, Germany and Turkey, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>L. sericata</i> (UK)	<i>L. sericata</i> (DE)	<i>L. sericata</i> (TR)
1	C23	ND	0.63±0.36	1.03±1.02
2	C25	1.82±0.46	2.12±0.58	3.00±2.53
3	11+13-MeC25	0.70±0.30	0.97±0.27	1.79±1.95
4	7-MeC25	ND	ND	0.57±0.21
5	3-MeC25	ND	0.58±0.08	0.50±0.17
6	C26	0.87±0.16	0.78±0.07	1.04±0.56
7	4-MeC26	ND	1.22±0.24	1.86±2.05
8	3-MeC26	0.64±0.24	ND	ND
9	Heptacosene*	ND	0.53±0.22	ND
10	Heptacosene*	0.78±0.67	1.86±0.83	0.77±0.77
11	C27	10.31±1.80	9.37±0.77	11.44±5.14
12	13+15-MeC27	4.54±1.59	6.77±1.29	8.19±7.14
13	7-MeC27	0.60±0.10	0.59±0.13	0.86±0.33
14	5-MeC27	0.54±0.14	0.51±0.09	0.50±0.11
15	4-MeC27	ND	0.60±0.08	0.57±0.24
16	3-MeC27	4.95±0.39	4.05±0.73	4.15±1.15
17	C28	1.60±0.23	1.20±0.18	2.52±0.84
18	13+15-MeC28	ND	0.60±0.23	ND
19	11+13-MeC28	2.64±0.43	2.43±0.87	1.98±0.82

Peak No.	Peak ID	<i>L. sericata</i> (UK)	<i>L. sericata</i> (DE)	<i>L. sericata</i> (TR)
20	3-MeC28	6.16±2.59	ND	6.27±3.25
21	Nonacosene*	ND	8.59±1.12	1.68±2.88
22	Nonacosene*	ND	4.51±0.98	ND
23	C29	9.28±1.50	3.89±0.46	4.84±1.23
24	13+15-MeC29	ND	3.60±1.01	3.73±2.66
25	11+13-MeC29	21.91±2.55	15.33±3.56	14.81±4.33
26	7-MeC29	3.44±0.51	1.86±0.46	2.24±1.20
27	4-MeC29	ND	2.60±1.07	2.74±0.91
28	3-MeC29	1.71±0.18	1.43±0.17	1.72±0.68
29	C30	0.50±0.08	1.71±0.30	1.01±0.43
30	8-MeC30	2.80±0.43	ND	ND
31	4-MeC30	ND	ND	1.85±0.80
32	3-MeC30	5.81±1.76	ND	ND
33	C31	4.98±0.75	9.17±2.00	7.81±2.62
34	13+15-MeC31	6.16±2.00	4.37±1.32	ND
35	11+13-MeC31	3.80±0.54	3.53±0.73	3.27±1.31
36	9+11-MeC31	ND	2.80±0.52	4.83±1.17
37	3-MeC31	ND	0.51±0.31	2.78±0.91
38	Dotriacontene*	0.68±0.09	ND	ND
39	C32	0.61±0.14	0.58±0.09	ND
40	C33	0.60±0.12	0.54±0.12	ND

ND Not detected

* Double bond positions were not determined

A total of 26 CHCs were detected in the CHC profiles of adult flies collected from the UK, 33 CHCs were detected in those collected from Germany, and 30 CHCs were detected in those collected from Turkey. The distribution according to the detected CHCs classes is shown in Table 7.2. Similar numbers of n-alkanes, alkenes and branched methyl alkanes were detected in the populations. Branched methyl alkanes were more abundant in all populations. It was seen on the plot that the populations had almost similar total percentage concentrations of n-alkane. The key distinguishing feature between the populations was the percentage concentrations of branched methyl alkanes and alkenes. While the percentage concentrations of branched methyl alkanes were higher in *L. sericata* adult flies collected from the UK and Turkey, alkene concentration was significantly higher in the German population.

Table 7.2: CHC composition showing the number and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *L. sericata* adult flies collected from the UK, Germany and Turkey

	<i>L. sericata</i> (UK)		<i>L. sericata</i> (DE)		<i>L. sericata</i> (TR)	
	number	Total % peak area	number	Total % peak area	number	Total % peak area
n-alkanes	9	31	10	30	8	33
Branched methyl HC	15	67	19	54	20	65
Alkenes	2	2	4	16	2	2
Total	26	100	33	100	30	100

GC chromatograms of *L. sericata* adult flies collected from three different countries are given in Figure 7.2. The chromatograms were similar in general appearance. Basically, this result supports the statement that the CHC profile, which has an unchanging and universal pattern except for some differences in the peak areas, is like a fingerprint.

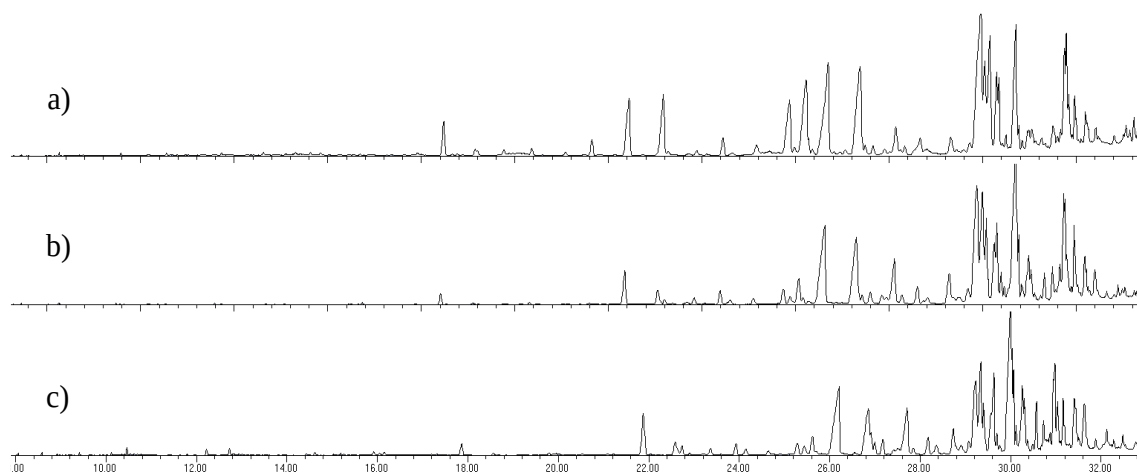


Figure 7.2: GC chromatograms showing CHC profiles of *L. sericata* adult flies collected from a) Turkey, b) Germany, c) the UK

The plot given in Figure 7.3 was created to observe the changes in the CHC profiles of the detected n-alkanes in detail. The predominant n-alkanes in the populations of the three countries were odd-numbered CHCs chains containing from 25 to 31 carbon atoms. C27:H was the predominant n-alkane in all three populations. In the UK population, C27:H and C29:H were in higher concentrations, while C31:H was less abundant. In the German population, C27:H and C31:H were in higher concentrations, whereas C29:H was less abundant. There was a similar situation with the German population in the Turkish population. When examined in terms of n-alkanes, the populations of Germany and Turkey show similarities, while the UK population differs from the other two populations.

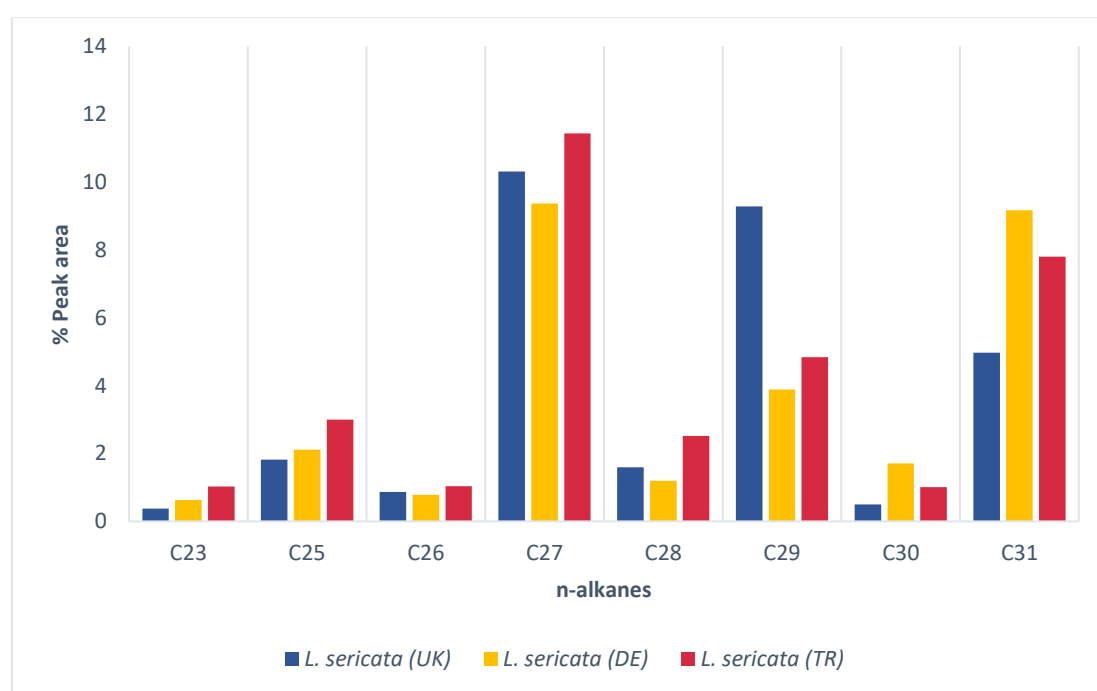


Figure 7.3: Plot showing the percentage concentrations of n-alkanes detected in *L. sericata* adult flies collected from the UK, Germany and Turkey

In order to examine the CHC profiles of the populations in terms of branched methyl alkanes, the plot showing the percentage concentrations of branched methyl alkanes is given in Appendix 35. The predominant branched methyl alkane common to all three populations was 11+13-MeC29. While the percentage concentration of this compound was nearly identical in the German and Turkish populations, the percentage concentration in the UK population was 5% higher. In addition, 13+15-MeC29, 4-MeC29, 9+11-MeC31 and 3-MeC31 were detected in both German and Turkish populations but not in the UK population. 3-MeC28 was detected only in UK and Turkish populations, and 13+15-MeC31 was detected only in the UK and German populations. 8-MeC30 and 3-MeC30 were detected only in the UK population, while 4-MeC30 was detected only in the German population.

Although the chemical profiles for adult flies from the three populations were similar, there were population-specific differences in the proportions of the CHC profiles extracted. Hence, PCA calculations were applied using the CHCs data given in Table 7.1 to visualise the results and distinguish between the populations statistically. PCA was performed using six principal components, describing 97.7% of the dataset variation, with the first three principal components as 87.8, 6.7 and 3.3%, respectively. 3D plots were used to clearly visualise the separation between clusters. In order to form the PCA plot given in Figure 7.4, PC2, PC3 and PC4 scores were preferred, where the separation between the clusters was more clearly achieved.

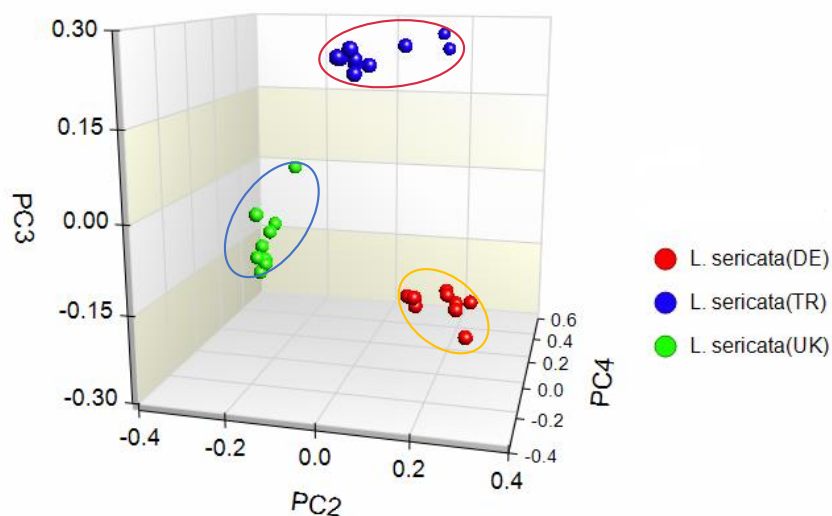


Figure 7.4: PCA plot showing PC3 vs PC2 vs PC4 for *L. sericata* adult flies collected from the UK, Germany and Turkey, using n-alkanes, alkenes and methyl branched alkanes; clusters were grouped by populations

Discriminant analysis was performed to determine which CHCs were most responsible for separating the UK, German and Turkish *L. sericata* adult fly populations. Among all the CHCs given in Table 7.1, the program identified 3-methyloctacosane, 3-methylhentriacontane, 13+15-methylhentriacontane and nonacosene as the most important CHCs in the discriminant analysis.

The results revealed qualitative and quantitative differences between populations. PCA analysis showed that samples were separated according to the populations (Figure 7.4). As can be easily seen on the plot created using PCA data, sample clusters representing the UK, German and Turkish populations of *L. sericata* adult flies were separated from each other. The differences in CHC profiles among the *L. sericata* populations analysed here reflect geographical diversity. The tight distribution of the repeat samples forming the clusters on the plot also showed that the variation and reproducibility of the results were high. There was no overlap between the three populations.

7.2.1.2 *C. vicina*

The CHCs identified in the CHC profiles of *C. vicina* adult flies were extracted on Day 1 after the pupae stages in the UK, Germany and Turkey are given in Table 7.3. The chain lengths of the detected CHCs range from C23:H to C31:H. CHCs with concentrations below 0.5% were not included for inter-population comparison.

Table 7.3: Compounds extracted from adult flies of *C. vicina* collected from the UK, Germany and Turkey, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>C. vicina</i> (UK)	<i>C. vicina</i> (DE)	<i>C. vicina</i> (TR)
1	C23	0.92±0.34	0.68±0.14	1.42±1.05
2	7-MeC23	ND	ND	0.85±0.62
3	C24	ND	ND	0.79±0.59
4	3-MeC25	1.70±1.15	ND	ND
5	C25	3.50±1.58	2.81±0.54	4.32±1.65
6	11+13-MeC25	3.29±1.24	1.57±0.41	4.71±2.97
7	9-MeC25	ND	ND	1.38±0.40
8	7-MeC25	1.70±2.09	0.68±0.17	1.09±0.26
9	5-MeC25	0.56±0.18	0.50±0.23	1.80±1.05
10	4-MeC25	ND	ND	0.83±0.50
11	3-MeC25	1.66±0.64	1.28±0.32	ND
12	C26	1.12±0.47	1.33±0.30	1.39±0.31
13	13+15-MeC26	1.28±0.33	1.24±0.49	ND
14	11+13-MeC26	1.09±0.23	0.91±0.32	ND
15	8-MeC26	0.87±0.57	0.58±0.24	ND
16	5-MeC26	ND	3.18±0.49	ND
17	4-MeC26	5.16±3.13	0.64±0.16	5.26±3.13
18	3-MeC26	0.58±0.08	0.71±0.42	1.30±1.31
19	Heptacosene*	ND	ND	0.62±0.18
20	C27	11.13±6.11	10.99±1.78	11.03±2.76
21	13+15-MeC27	0.53±0.16	0.72±0.55	ND
22	11+13-MeC27	9.77±3.10	9.94±3.75	11.73±2.54
23	7-MeC27	4.41±7.72	2.59±0.45	ND
24	6-MeC27	1.06±0.99	ND	ND
25	5-MeC27	2.10±0.96	2.47±0.51	ND

Peak No.	Peak ID	<i>C. vicina</i> (UK)	<i>C. vicina</i> (DE)	<i>C. vicina</i> (TR)
26	4-MeC27	1.10±0.76	2.32±2.18	2.23±0.89
27	3-MeC27	5.47±1.89	6.22±1.42	1.38±0.59
28	C28	1.54±0.73	2.07±1.30	2.63±0.76
29	13+15-MeC28	2.23±0.67	3.62±0.77	ND
30	11+13-MeC28	2.09±0.94	2.10±0.43	ND
31	8-MeC28	1.56±1.03	1.52±0.44	ND
32	6-MeC28	1.13±0.62	1.50±1.64	ND
33	4-MeC28	5.61±3.96	4.90±1.14	5.00±1.57
34	3-MeC28	1.98±0.95	3.06±2.38	3.89±2.65
35	C29	6.51±3.42	6.20±2.16	9.06±2.56
36	13+15-MeC29	1.40±0.73	2.31±2.54	ND
37	11+13-MeC29	6.50±3.25	7.71±1.11	2.77±0.87
38	7-MeC29	1.37±0.24	1.95±0.31	8.36±2.45
39	5-MeC29	ND	0.58±0.30	ND
40	4-MeC29	0.92±0.50	0.82±0.27	1.12±0.40
41	3-MeC29	1.82±0.90	2.19±0.55	3.99±1.91
42	C30	0.70±0.28	0.50±0.20	0.82±0.21
43	8-MeC30	1.15±0.69	1.11±0.44	2.33±0.83
44	4-MeC30	0.97±0.69	1.10±0.48	3.46±0.98
45	3-MeC30	0.66±0.21	0.53±0.43	ND
46	C31	0.57±0.23	0.82±0.40	1.59±0.72
47	13+15-MeC31	0.52±0.24	0.65±0.22	ND
48	11+13-MeC31	ND	0.56±0.19	ND
49	9+11-MeC31	0.95±0.43	1.61±0.55	1.60±0.64
50	3-MeC31	0.53±0.20	1.20±0.46	1.25±0.37

ND Not detected

* Double bond positions were not determined

A total of 41 CHCs were detected in the CHC profiles of *C. vicina* adult flies collected from the UK, A total of 43 CHCs were detected in Germany, and 31 CHCs were detected in those collected from Turkey. The numerical distribution of the compounds according to the CHCs classes is given in Table 7.4. The numbers of n-alkanes and branched methyl alkanes detected in the UK and German populations were equal. However, although the same number of n-alkanes was detected in the Turkish population, fewer branched methyl alkanes were detected there compared to the other two populations. It was understood that the percentage concentrations of branched methyl alkanes in the chemical profiles of adult flies were much higher than the n-alkane concentrations. Although the same number of n-alkanes was detected in the populations, the total percentage concentrations of n-alkanes detected in the Turkish population were higher than in others. The UK and German populations already had more branched methyl alkanes, and the total percentage concentration of branched methyl alkanes was greater than in Turkey. On the other hand, only heptacosene, with a 1% concentration, was included in the Turkish population.

Table 7.4: CHC composition showing the number and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *C. vicina* adult flies collected from the UK, Germany and Turkey

	<i>C. vicina</i> (UK)		<i>C. vicina</i> (DE)		<i>C. vicina</i> (TR)	
	number	Total % peak area	number	Total % peak area	number	Total % peak area
n-alkanes	8	26	8	25	9	33
Branched methyl HC	33	74	35	75	21	66
Alkenes	-	-	-	-	1	1
Total	41	100	43	100	31	100

Observing the chromatograms of the populations (Figure 7.5), the chemical profiles of the UK and German populations were similar. However, the percentage concentrations of CHCs with shorter carbon chains in the chemical profile of the Turkish population differed from the other populations. Also, it was observed that the Turkish population had more CHCs with longer carbon chains CHCs in their profiles. Thus, although there were differences in the percentage concentrations of compounds between chromatograms, it was clear that the CHC profiles of the *C. vicina* species were conserved in a unique pattern such as fingerprinting across populations.

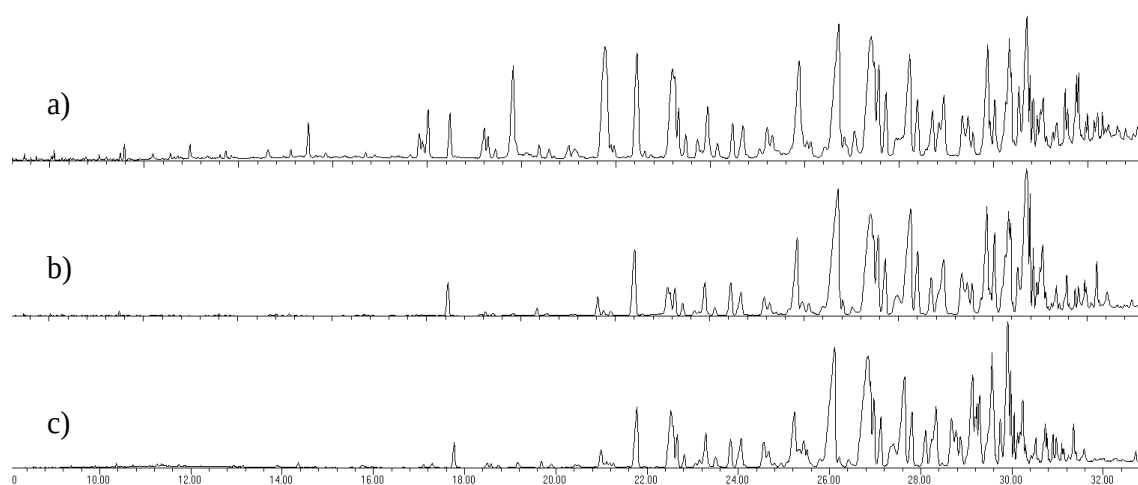


Figure 7.5: GC chromatograms showing CHC profiles of *C. vicina* adult flies collected from a) Turkey, b) Germany, c) the UK

In the CHC profiles of all three populations, n-alkanes with higher densities compared to the others were n-alkanes with an odd number of carbons (Figure 7.6). Another common denominator in the given populations was that C27:H was the predominant n-alkane, and its percentage concentration was the same in all three populations. The difference that emerged was that the percentage concentrations of n-alkanes detected in the Turkish population were considerably higher than those in the UK and German populations.

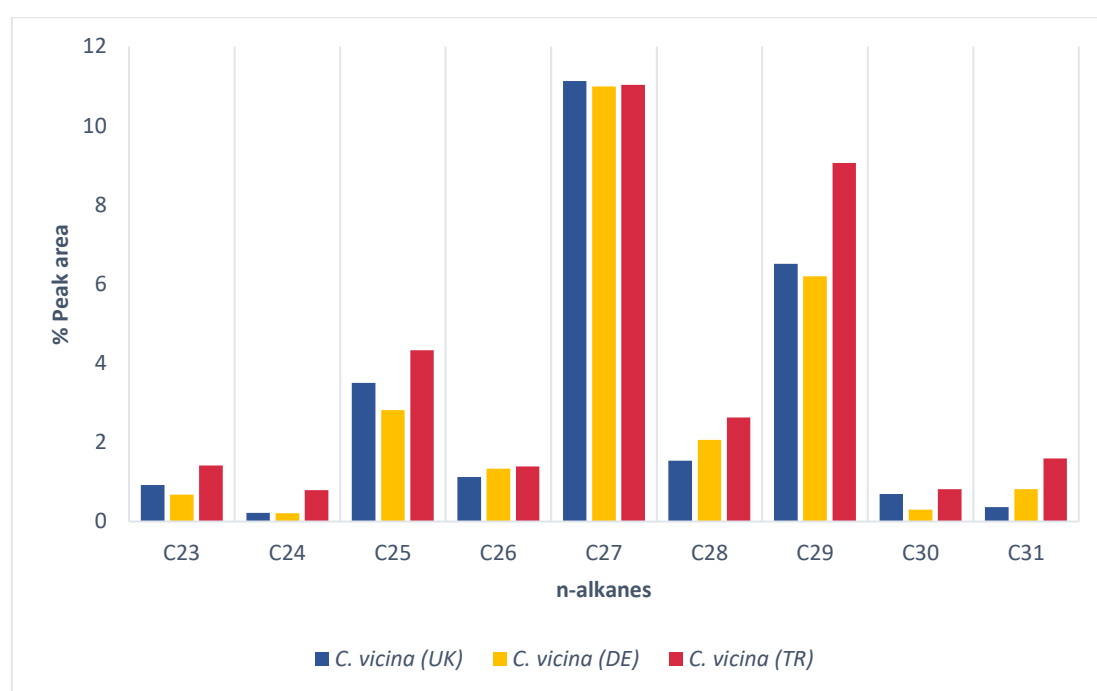


Figure 7.6: Plot showing the percentage concentrations of n-alkanes detected in *C. vicina* adult flies collected from the UK, Germany and Turkey

When the branched methyl alkane compounds of the populations and their concentrations were examined (Appendix 36), the specific branched methyl alkanes were 9-MeC25 in the Turkish population, 3-MeC25 in the UK population, and 5-MeC26 in the German population. 11+13-MeC29 was the most predominant branched methyl alkane in all populations. However, differences in its concentration may be used to distinguish populations. In the UK and German populations, which have almost similar CHC profiles, 3-MeC27 and 11+13-MeC29 have higher but different quantities concentrations than other branched methyl alkanes, while 7-MeC27 has a higher concentration in the Turkish population.

Although similarities and differences were revealed based on the CHC profiles of *C. vicina* adult flies collected from the UK, German and Turkish populations, PCA calculations were applied using the CHCs data given in Table 7.3 to visualise the results to separate the populations statistically. PCA was performed using six principal components, describing 95.7% of the dataset variation, with the first three principal components being 86.1, 7.0 and 2.6%, respectively. The 3D plot has been preferred so that the separation between clusters can be seen more clearly. In order to form the PCA plot given in Figure 7.7, PC2, PC3 and PC4 scores were preferred, where the separation between the clusters was more clearly achieved.

Discriminant analysis was performed to determine which CHCs were most responsible for separating *C. vicina* adult fly populations of the UK, Germany and Turkey. Among all the CHCs given in Table 7.3, the program identified 4-methylhexacosane, 3-methylheptacosane, 7-methylheptacosane and 3-methyloctacosane as the most important CHCs in the discriminant analysis.

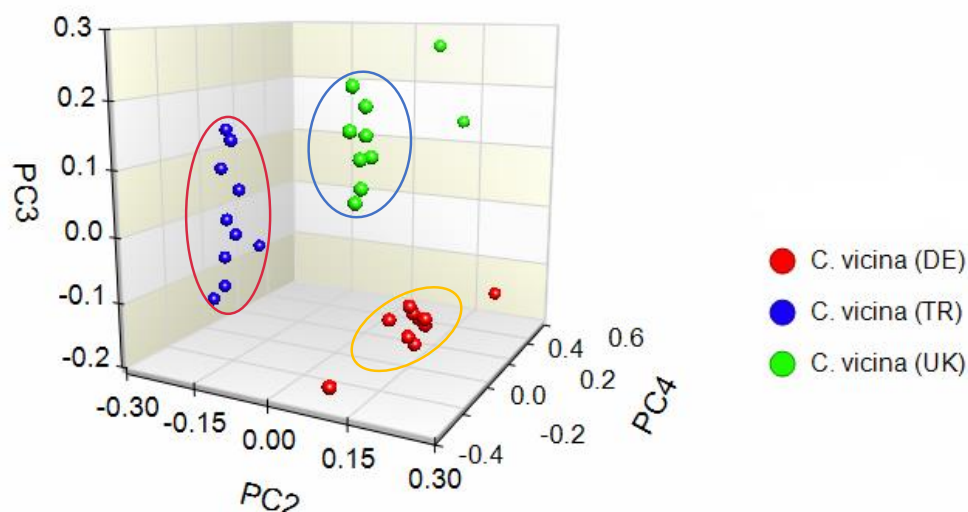


Figure 7.7: PCA plot showing PC3 vs PC2 vs PC4 for *C. vicina* adult flies collected from the UK, Germany and Turkey, using n-alkanes, alkenes and methyl branched alkanes; clusters were grouped by populations

The results revealed qualitative and quantitative differences between populations. PCA analysis showed that samples were separated according to the populations. According to the UK, German and Turkish populations, the PCA plot shows that adult flies of *C. vicina* were separated and clustered themselves. Although indicators provide similarity and differentiation based on the CHC profiles, it becomes difficult to distinguish as the population and number of samples increase. In this case, PCA stands out as an easy application to distinguish the clusters. The similarity in the GC chromatograms showing the CHC profiles from different species populations is evident (Figure 7.7). While it is impossible to distinguish between the UK and German populations visually, using the CHC analysis and PCA, it was revealed that there were detectable differences even between populations with very similar chemical profiles. There was no overlap between the three populations. The differences in CHC profiles among the populations analysed reflect geographical diversity.

7.2.2 Discussion of Adult Fly Stage Comparison

Although there are some CHCs with lower boiling points, the CHC profiles of adult flies are dominated by CHCs with higher boiling points (with larger retention times, 20 and 32 minutes) compared to larval and puparial stages. Adult flies must be able to withstand much drier environmental conditions, so cuticles need larger carbon chains that offer significant impermeability [40]. The resistance of this barrier to desiccation is mainly affected by the chain length, degree of saturation and degree of branching of CHCs [94].

The percentage concentrations of branched methyl alkanes were higher in adult flies compared to larval and puparial stages. Furthermore, the highest alkene concentration was detected in adult flies when compared to other life stages, where alkene compounds were generally detected in either very low concentrations or absent. This may be an indication that adult flies have a greater need for communication with nestmates. Compounds with lower melting points and volatility, such as alkenes and branched alkanes, play a critical role in communication; alkenes, in particular, appear to be essential for signal exchange during chemical communication and sex determination, although they are present in lower concentrations than other CHCs classes. Therefore, insects have to synthesise unsaturated or branched alkanes, although they tend to lower the melting

temperature [97]. Furthermore, the increased permeability of surface lipids at higher ambient temperatures may result from the need for interaction with colony members [86].

Using CHC with PCA analyses, it was possible to distinguish separate geographical populations for *L. sericata* and *C. vicina* adult flies based on their chemical profiles. These results show that answering questions about whether a corpse has been relocated in a criminal case is possible.

In Chapter 4, the CHC analysis for species identification was demonstrated by comparing the CHC profiles of adult flies of *L. sericata* and *C. vicina* species. The results obtained in this section proved that the *L. sericata* species has a CHC profile like a fingerprint and continues to carry the CHC profiles in a general pattern, even if there is a geographical region and population change and each population changes qualitatively or quantitatively, depending on the environmental conditions to which they are exposed. The Turkish population reared at higher temperatures contained higher proportions of n-alkanes than other CHCs because n-alkanes are more protective against desiccation. In addition, geographical distances also have an impact on the chemical profiles. The results of Byrne et al. [166] with *P. regina* adult flies showed differences between the CHC profiles of both close and distant populations.

The results obtained in this section of the chapter were also similar, supporting chemical differences between populations of both *L. sericata* and *C. vicina* adult flies in close-range and long-range geographies. In comparison, there are more similarities between the CHC profiles of the populations of the UK and Germany, which have similar climatic characteristics and are closely distant between them. In contrast, the distance between them and the population of Turkey is higher, and the climate of Turkey is different from these two countries. This situation explains that the Turkish population is different from the other two populations. The fact that species reared in humid climates in the UK have more alkenes and branched methyl alkanes than species in drier habitats in Turkey can be explained by the different waterproofing capacities of these compounds.

7.3 Empty Puparia Stage

Pupae have an excellent lipid membrane that functions as a hydrophobic barrier. Toolson [89] determined the effects of larval and puparial thermal regimes on transcuticular water loss rates and epicuticular composition in adult flies of *Drosophila pseudoobscura*, finding that the cuticle structure of adult flies was shaped according to the temperatures they were exposed to during the puparial period, although they were exposed to different temperatures during the larval and puparial stages. With this study, the importance of the pupae period for determining the crime scene and ambient temperatures has been understood. Empty puparia can offer undeniable evidence when investigating crimes that happened a long time ago when living forensic entomological evidence cannot be found.

Despite advances in the use of empty puparia in species identification and age estimation, until recently, there has been no research on the differentiation between populations of the CHC profiles of the same empty puparia species. In addition, although it is known that CHC profiles differ according to environmental and temperature variables, the potential role of empty puparia CHC profiles in climatic adaptation has not been extensively studied. In this study, the variation between populations of *C. vicina* and *L. sericata* empty puparia collected from the UK, Germany and Turkey was determined based on the qualitative and quantitative differences in CHC profiles extracted from the epicuticle. CHC compounds were analysed by GC-MS, and multivariate statistical analysis was applied to the results to distinguish between species.

7.3.1 Results for Empty Puparia Stage

7.3.1.1 *L. sericata*

The empty puparia left behind by adult flies of *L. sericata* hatching from the puparial stage in the UK, Germany and Turkey were collected, and the CHC profiles were extracted. The CHCs compounds detected in the chemical profiles of the populations are given in Table 7.5. The chain lengths of the detected CHCs range from C23:H to C33:H.

Table 7.5: Compounds extracted from empty puparia of *L. sericata* collected from the UK, Germany and Turkey, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>L. sericata</i> (UK)	<i>L. sericata</i> (DE)	<i>L. sericata</i> (TR)
1	C23	ND	ND	0.68±0.21
2	C24	ND	ND	0.63±0.18
3	C25	7.16±1.32	1.32±0.20	1.76±0.39
4	C26	0.78±0.22	0.83±0.15	1.01±0.21
5	C27	16.97±3.77	23.28±4.89	18.37±4.87
6	11+13-MeC27	ND	1.10±1.33	0.53±0.25
7	3-MeC27	2.94±0.80	3.18±0.67	2.25±0.74
8	C28	2.55±0.50	3.27±0.55	4.53±1.38
9	4-MeC28	ND	ND	1.12±0.14
10	3-MeC28	1.41±0.56	1.79±0.56	ND
11	Nonacosene*	ND	2.94±2.15	ND
12	C29	24.18±4.97	31.31±5.56	30.45±9.60
13	13+15-MeC29	ND	ND	3.90±0.99
14	11+13-MeC29	5.73±2.04	6.74±1.88	ND
15	7-MeC29	1.12±0.29	1.26±0.30	ND
16	4-MeC29	1.00±0.26	1.01±0.28	1.22±0.46
17	3-MeC29	0.84±0.20	1.13±0.34	1.79±0.76
18	C30	1.36±0.37	2.01±0.38	3.74±1.20
19	4-MeC30	0.60±0.17	0.86±0.31	0.67±0.20
20	3-MeC30	1.49±0.60	ND	6.40±2.10
21	C31	10.02±2.61	12.73±2.33	13.47±3.99
22	13+15-MeC31	ND	2.23±0.55	ND
23	11+13-MeC31	2.68±0.84	1.35±0.39	ND
24	9+11-MeC31	ND	ND	1.58±0.32
25	3-MeC31	ND	ND	1.28±0.60
26	C32	ND	ND	1.12±0.20
27	C33	1.13±0.73	1.66±0.42	3.51±0.95

ND Not detected

* Double bond positions were not determined

The compounds detected were arranged according to CHCs classes and summarised in Table 7.6 to show their numerical distribution according to the populations. The least number of CHCs, 17 in total, were detected in the UK population. A total of 19 CHCs were detected in the German population. The total concentrations of n-alkanes were higher in all three populations, and the total percentages of CHCs between populations were quite similar. The largest number of CHCs, which was 21, were detected in the Turkish population. Although the number of branched methyl alkanes detected in the populations was almost similar, the number of n-alkanes detected in the Turkish population was higher than in the others. While eight n-alkanes were detected in the UK and German populations, eleven were detected in the Turkish population. Adequate alkene concentrations could not be detected in the UK and Turkish populations; only nonacosene was found in the German population, with a 3% concentration.

Table 7.6: CHC composition showing the number and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *L. sericata* empty puparia collected from the UK, Germany and Turkey

	<i>L. sericata</i> (UK)		<i>L. sericata</i> (DE)		<i>L. sericata</i> (TR)	
	number	Total % peak area	number	Total % peak area	number	Total % peak area
n-alkanes	8	78	8	76	11	79
Branched methyl HC	9	22	10	21	10	21
Alkenes	-	-	1	3	-	-
Total	17	100	19	100	21	100

The chemical profiles of *L. sericata* empty puparia collected from the UK, Germany and Turkey, whose GC chromatograms are given in Figure 7.8, were similar except for the differences in the peak areas of the compounds. While the CHC profiles of the UK and German populations were quite similar, the presence of CHCs with longer carbon chains (higher retention times, 31 to 33 minutes) in the Turkish population created a difference.

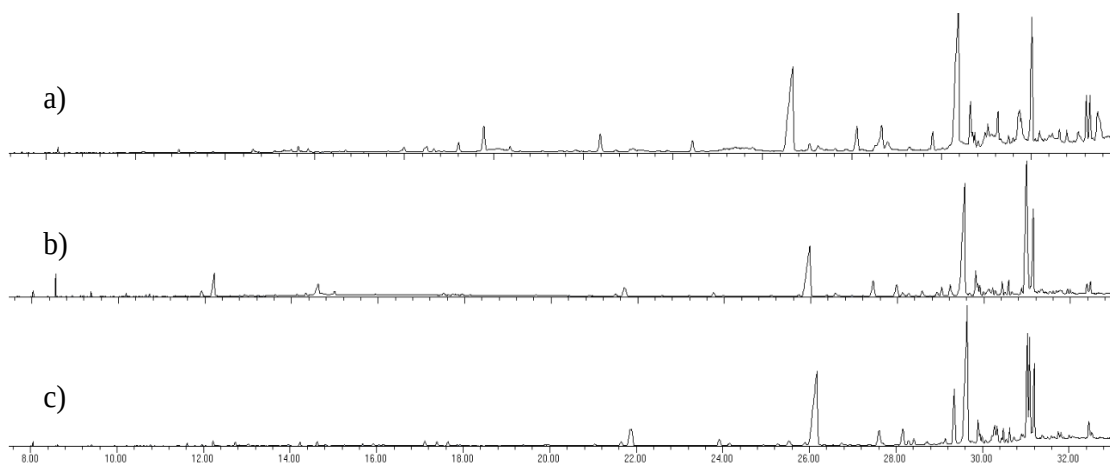


Figure 7.8: GC chromatograms showing CHC profiles of *L. sericata* empty puparia collected from a) Turkey, b) Germany, c) the UK

Although close numbers of branched methyl alkanes were detected in the populations, the percentage concentrations, carbon numbers and isomers of the branched methyl alkanes detected differed (Appendix 37). While the UK and German populations had more common branched methyl alkanes, more individual branched methyl alkanes were detected in the Turkish population. 13+15-MeC31 was detected only in the German population. 4-MeC28, 13+15-MeC29, 9+11-MeC31 and 3-MeC31 were detected only in the Turkish population. 11+13-MeC29 was detected in the UK and German populations and was the predominant branched methyl alkane detected in these populations. 3-MeC30 was detected in both the UK and Turkish populations. However, while it was the predominant branched methyl alkane detected in the Turkish population, it was relatively lower in the UK population. The presence/absence of branched methyl alkanes CHCs profiles or differences in concentrations between populations can be considered a distinguishing indicator.

While the total percentage concentrations of n-alkanes were nearly the same between populations, the percentage concentrations of n-alkanes varied between populations. The n-alkane concentrations according to the populations are shown in Figure 7.9. In the CHC profiles of the populations, the odd-numbered CHC chains containing from 25 to 33 carbon atoms were the predominant n-alkanes. C29:H was the predominant n-alkane for all three populations, while its percentage concentration was almost similar. A similar situation was valid for the C31:H n-alkane. The most significant difference between populations was obtained in the percentage concentrations of C25:H and C27:H n-alkanes. The percentage concentrations of C25:H detected in the UK population were almost three times greater than in the other two populations. The highest concentrations of C27:H was detected in the German population and the lowest in the Turkish population. In addition, the detection concentrations of n-alkanes with carbon numbers C28:H and C33:H were higher in the Turkish population than in the other two populations, which also emerged as a distinguishing indicator between the populations.

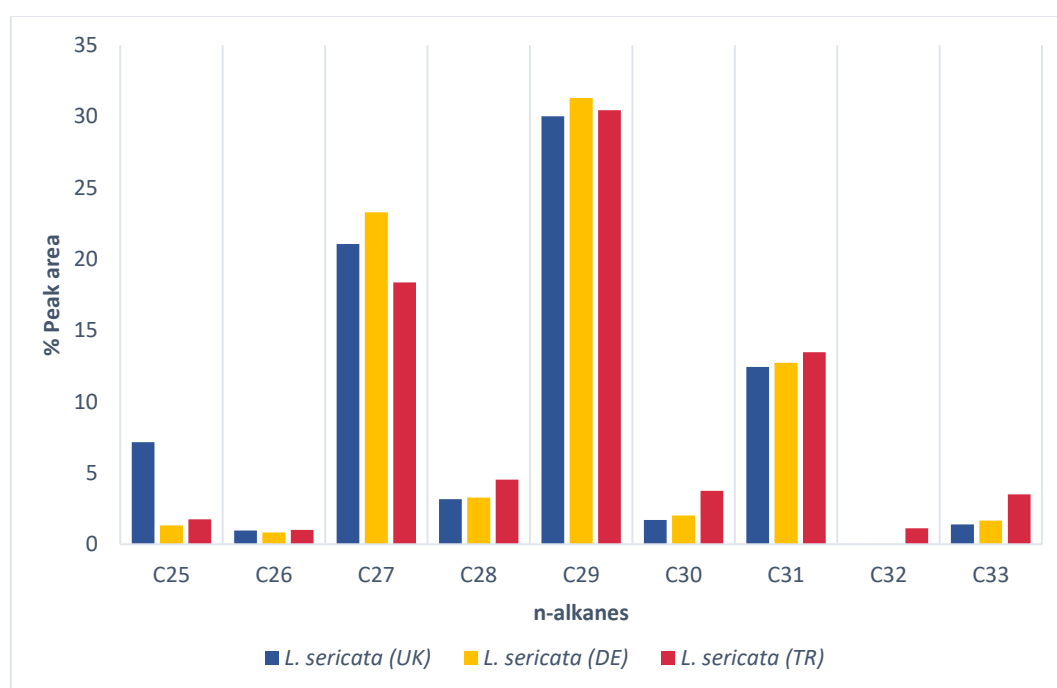


Figure 7.9: Plot showing the percentage concentrations of n-alkanes detected in *L. sericata* empty puparia collected from the UK, Germany and Turkey

Although similarities and differences were explained for the CHC profiles of *L. sericata* empty puparia collected from the UK, German and Turkish populations, PCA calculations were applied using the CHCs data given in Table 7.6 to visualise the results and to distinguish between the populations statistically. PCA was performed using six principal components, describing 99.1% of the dataset variation, with the first three principal components as 88.6, 8.8 and 1.7%, respectively. The 3D plot was used to clearly visualise the separation between clusters. In order to form the PCA plot given in Figure 7.10, PC2, PC3 and PC4 scores were preferred, where the separation between the clusters was more clearly achieved. PCA was used to examine inter-population CHC profile variation and evaluate the effects of geographical differences on CHC profiles.

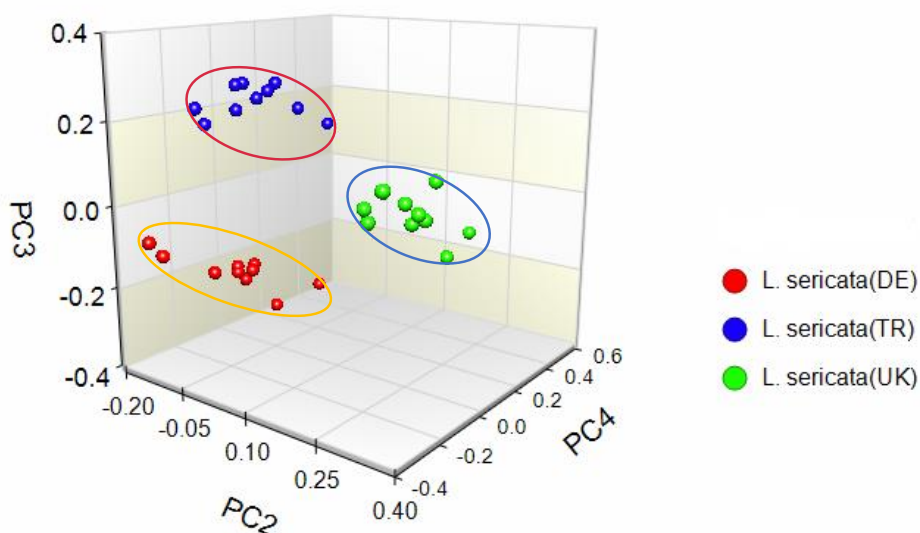


Figure 7.10: PCA plot showing PC3 vs PC2 vs PC4 for *L. sericata* empty puparia collected from the UK, Germany and Turkey, using n-alkanes, alkenes and methyl branched alkanes; clusters were grouped by populations

Discriminant analysis was performed to determine which CHCs were most responsible for separating populations of the UK, German and Turkish *L. sericata* empty puparia. Among all the CHCs, given in Table 7.6, the program identified pentacosane (C₂₅:H),

nonacosane (C29:H), and 3-methylhentriacontane, 11+13-methylnonacosane as the most important CHCs in the discriminant analysis.

L. sericata empty puparia collected from the UK, German and Turkish populations on the PCA plot were clustered based on the differences in their CHC profiles. The differences in CHC profiles among the *L. sericata* populations from the three countries analysed here reflect geographical diversity. The clusters were separate from each other and showed a tight distribution. There was no overlap between the three populations. This distinction between the obtained populations is promising, especially given similar chemical profiles of the UK and German populations. The results obtained, just as encountered in other life cycle stages, were found to have a unique pattern, such as a fingerprint on the empty puparia structures. This pattern was different enough to distinguish between populations.

7.3.1.2 *C. vicina*

The empty puparia left behind by adult flies of *C. vicina* hatching from the puparial stage in the UK, Germany and Turkey were collected. The CHCs were detected by GC-MS analysis to be a mixture of n-alkanes, alkenes and branched methyl alkanes. The CHCs compounds identified on the chromatograms are given in Table 7.7. The chain lengths of the detected CHCs range from C21:H to C32:H. CHCs with concentrations below 0.5% were not included for inter-population comparison.

Table 7.7: Compounds extracted from empty puparia of *C. vicina* collected from the UK, Germany and Turkey, total percentage of each compound present and percentage standard deviation

Peak No.	Peak ID	<i>C. vicina</i> (UK)	<i>C. vicina</i> (DE)	<i>C. vicina</i> (TR)
1	3-MeC21	1.44±1.11	ND	ND
2	C23	ND	0.87±0.49	0.83±0.09
3	C24	ND	ND	0.55±0.07
4	C25	4.52±0.87	31.15±6.66	4.96±0.42
5	3-MeC25	0.57±0.11	0.51±0.21	0.73±0.13
6	C26	3.74±1.34	1.70±0.50	2.23±0.16
7	3-MeC26	0.93±0.35	0.57±0.17	0.90±0.21
8	C27	36.66±8.55	23.44±5.36	32.02±2.73
9	11+13-MeC27	4.19±1.01	1.97±0.76	3.04±0.68
10	7-MeC27	1.31±0.55	0.70±0.25	1.21±0.23
11	5-MeC27	1.13±0.25	0.61±0.20	0.98±0.21
12	4-MeC27	0.99±0.19	0.53±0.17	0.79±0.30
13	3-MeC27	8.13±1.45	5.69±1.68	7.50±1.36
14	C28	4.71±1.04	2.99±0.96	5.99±0.82
15	13+15-MeC28	1.21±0.46	1.06±0.20	ND
16	11+13-MeC28	1.37±0.22	ND	0.62±0.12
17	7-MeC28	0.58±0.15	ND	0.57±0.15
18	5-MeC28	1.74±0.36	ND	ND
19	4-MeC28	0.52±0.13	0.77±0.25	1.68±0.22
20	3-MeC28	2.24±0.62	ND	0.90±0.19
21	Nonacosene*	ND	4.70±1.21	ND
22	C29	14.10±3.44	9.03±2.26	21.34±2.85
23	13+15-MeC29	0.72±0.15	ND	1.27±0.21
24	11+13-MeC29	3.92±0.84	4.96±1.15	3.06±0.30
25	7-MeC29	1.18±0.26	0.87±0.28	0.94±0.13
26	4-MeC29	0.83±0.68	0.56±0.17	ND
27	3-MeC29	1.64±0.46	1.28±0.37	2.38±0.67
28	C30	0.56±0.12	0.51±0.12	1.66±0.26
29	3-MeC30	ND	1.23±3.08	ND
30	C31	0.96±0.41	1.78±0.59	3.82±0.76
31	11+13-MeC31	ND	0.53±0.17	ND
32	3-MeC31	ND	1.04±0.85	ND
33	3-MeC32	ND	0.95±0.38	ND

ND Not detected

* Double bond positions were not determined

The distribution of detected compounds by CHCs classes is given in Table 7.8. Similar numbers of CHC compounds were detected in the UK and German populations. However, fewer CHCs were detected in the Turkish population. While the number of detected n-alkanes was almost similar in these three 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. It was determined that n-alkanes were abundant and branched methyl alkanes were lower in all three populations. In the Turkish population with the highest n-alkane percentage concentration, 8% more n-alkane concentration was detected than in the UK, with the lowest percentage concentration. On the other hand, the branched methyl alkane detected in the UK population was the highest and was 11% higher than the German population, which had the lowest branched methyl alkane concentrations. Although adequate alkene concentrations could not be detected in the UK and Turkish populations, only nonacosene was detected, with 5% concentrations in the German population.

Table 7.8: CHC composition showing the number and percentage concentrations of n-alkanes, alkenes and branched methyl alkanes for *C. vicina* empty puparia collected from the UK, Germany and Turkey

	<i>C. vicina</i> (UK)		<i>C. vicina</i> (DE)		<i>C. vicina</i> (TR)	
	number	Total % peak area	number	Total % peak area	number	Total % peak area
n-alkanes	7	66	8	71	9	73
Branched methyl HC	19	34	17	24	15	27
Alkenes	-	-	1	5	-	-
Total	26	100	26	100	24	100

GC chromatograms, given in Figure 7.11, show the chemical profiles of *C. vicina* empty puparia collected from the UK, Germany and Turkey. The chromatograms were similar in general appearance. However, while the CHC profiles of the UK and German populations were quite similar, the percentage concentrations of CHCs in the Turkish population manifest a difference. This result supports the statement that the CHC profile is like a fingerprint; it does not change and has a universal pattern.

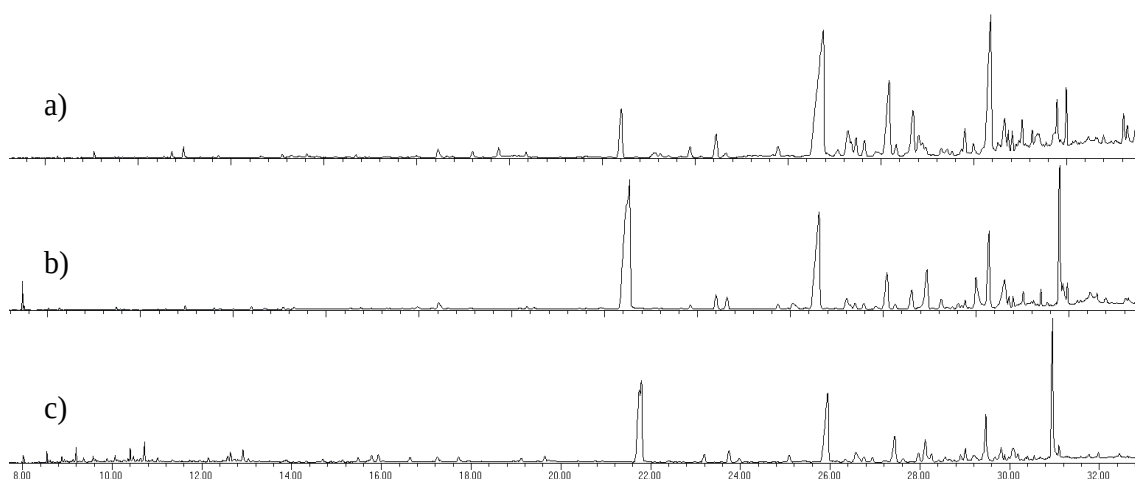


Figure 7.11: GC chromatograms showing CHC profiles of *C. vicina* empty puparia collected from a) Turkey, b) Germany, c) the UK

The variation of n-alkane concentration detected in CHC profiles according to populations is shown in the plot given in Figure 7.12. The predominant n-alkanes in the UK and Turkish populations were C27:H and C29:H. While the percentage concentration of C27:H detected in the UK population was higher than in the Turkish population, the percentage concentration of C29:H detected in the latter was higher than in the former. On the other hand, the predominant n-alkanes in the German population were C25:H and C27:H. The percentage concentration of C25:H detected in the German population was six times higher than that detected in the UK and Turkish populations.

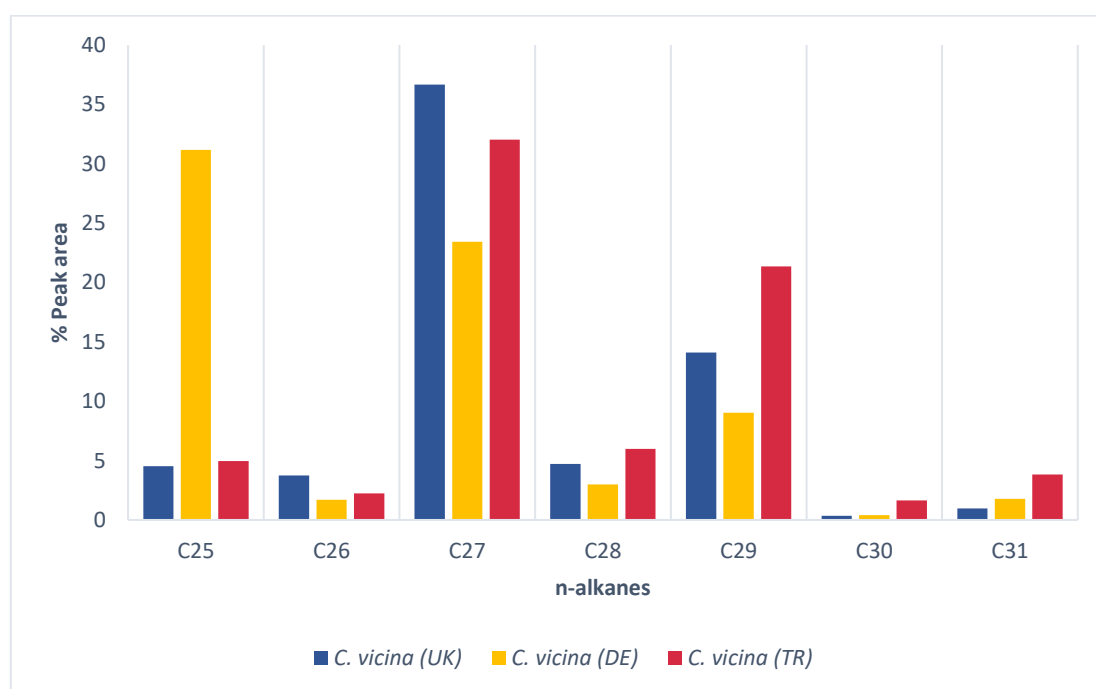


Figure 7.12: Plot showing the percentage concentrations of n-alkanes detected in *C. vicina* empty puparia collected from the UK, Germany and Turkey

When the percentage concentrations of branched methyl alkanes detected in the CHC profiles of empty puparia collected from the UK, Germany and Turkey were examined (Appendix 38), the predominant compounds in all three populations were the same: 3-MeC27, 11+13-MeC27 and 11+13-MeC29. However, differences were found in the percentage concentrations of these compounds. While a branched methyl alkane could not be detected only for the Turkish population, 3-MeC21 and 5-MeC28 were detected only in the UK population and 3-MeC30 and 3-MeC31 were detected only in the German population. Likewise, 13+15-MeC28 was detected only in the UK and German populations and 11+13-MeC28 and 13+15-MeC29 were detected only in the UK and Turkish populations. The presence/absence of branched methyl alkanes in structures or differences in their concentrations can be considered a distinguishing indicator between populations.

Although the chemical profiles for empty puparia from the three populations were similar, there were population-specific differences in the proportions of the CHC profiles extracted. Therefore, PCA calculations were applied using the CHCs data given in Table 7.7 to visualise the results and distinguish between the populations statistically. PCA was performed using six principal components, describing 99.4% of the dataset variation with the first three principal components being 85.4, 12.8 and 1.2%, respectively. The 3D plot has been preferred so that the separation between clusters can be seen more clearly. In order to form the PCA plot given in Figure 7.13, PC2, PC3 and PC4 scores were preferred, where the separation between the clusters was more clearly achieved. Using PCA to examine inter-population CHC profile variation aimed to evaluate the effects of geographical differences on the CHC profile.

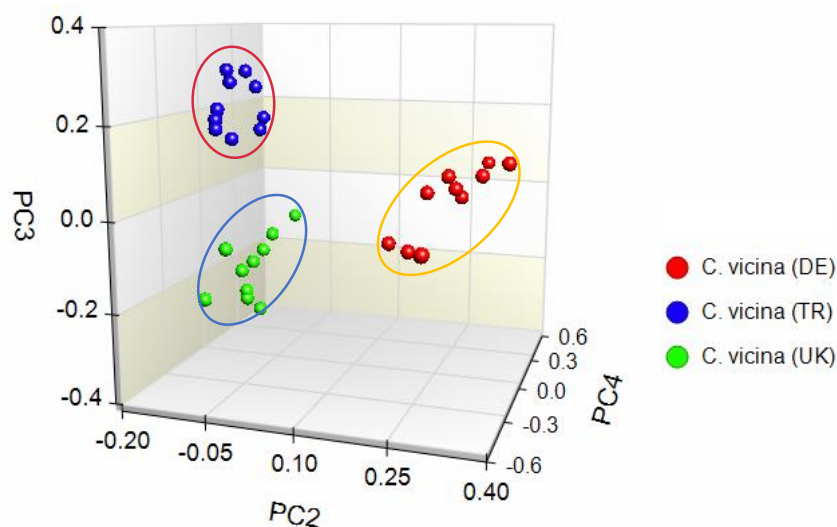


Figure 7.13: PCA plot showing PC3 vs PC2 vs PC4 for *C. vicina* empty puparia collected from the UK, Germany and Turkey, using n-alkanes, alkenes and methyl branched alkanes; clusters were grouped by populations

Discriminant analysis was performed to determine which compounds were most responsible for separating populations of the UK, German and Turkish *C. vicina* empty puparia. Among all the CHCs, given in Table 7.7, the program identified pentacosane (C25:H), heptacosane (C27:H) and nonacosane (C29:H) as the most important CHCs in the discriminant analysis.

The differences in CHC profiles among the *C. vicina* populations analysed show geographical diversity. As can be easily seen on the plot created using PCA data, sample clusters representing the UK, German and Turkish populations were clearly separated based on differences in their chemical profiles. The tight distribution of the repeat samples forming the clusters on the plot also showed that the variation and reproducibility of the results were high. This distinction between the obtained populations is promising, especially given similar chemical profiles of the UK and German populations. Furthermore, there was no overlap between the three populations. The results obtained in this section proved that the *C. vicina* species has a CHC profile like a fingerprint, which continues to carry the CHC profiles in a general pattern even if there is a geographical

region and population change. However, some variations in the structures allow distinguishing between species.

7.3.2 Discussion of Empty Puparia Stage Comparison

While pupae provide higher water resistance during the puparial stage, empty puparia can give information about the environmental conditions after the adult flies emerge. Waterproofing requirements change with temperature differences, so CHC profiles are expected to change to reflect the climatic conditions to which insects are exposed. In addition, the epicuticular composition affects cuticle water permeability, particularly the percentage concentrations of short and long-chain n-alkanes and branched methyl alkanes [92]. Therefore, chain length can be expected to increase with the increasing need for waterproofing. As a result, it was expected that n-alkanes, which provide higher water protection, were concentrated in CHC profiles.

Furthermore, alkenes and branched methyl alkanes, which are CHC classes with lower waterproofing properties, should be relatively abundant in species from more humid climates such as the UK. In comparison, n-alkanes with better waterproofing properties should be more abundant in drier climates such as Turkey. Branched methyl alkanes prevent tight molecular packing, which increases membrane fluidity and permeability. Organisms form a structure rich in n-alkanes and less in branched methyl alkane during the puparial stage when they need protection most, which indicates how much protection CHC profiles provide insects.

The identified similarities and differences in chemical profiles can be used to indicate the geographical origin of populations. Differences in environmental conditions between the UK, Germany and Turkey explain the differences in CHC compositions of the species. While the CHC profiles of empty puparia were relatively similar between the UK and German populations, the profile of the Turkish population differed from them. This may be the result of both the distance between populations and the climate difference.

7.4 Summary of Findings on Comparative Studies for the UK, German and Turkish Populations

There are questions about applying entomological data to death investigations, as studies have shown that developmental periods can vary with population and environmental factors, due to which they are considered less reliable in criminal investigations [180]. On the other hand, this research shows that identifications between species and age estimation of the species can be performed daily with data obtained from different populations using the CHC analysis, as indicated by the presented identification studies (Chapter 4), ageing studies (Chapter 5) and determination of the differences between the populations (Chapters 6 and 7). Differences between populations do not affect them. Moreover, questions about the crime scene and whether the corpse has been transported can be answered quickly. These studies are significant both in determining PMI_{min} and in guiding future research areas. By approaching research in this way, the forensic entomology community can address the cultural, legal and scientific prospects of the field from different perspectives.

This study was carried out using the first-generation adult flies and their empty puparia, which emerged by rearing eggs from *L. sericata* and *C. vicina* adult flies collected from the wild in the UK, Germany and Turkey. The primary purpose of this chapter was to use qualitative and quantitative analysis of chemical profiles to compare adult fly populations and their empty puparia. It was hypothesised that exposure of populations in the UK, Germany and Turkey to climatic thermal differences would lead to predictable, genetically based differences in CHC profiles between populations. Specifically, longer-chain CHCs were detected in samples collected from populations exposed to high-temperature rearing conditions in Turkey. Also, the differences in CHC profiles between populations maintained in heterogeneous climatic conditions in the field and controlled thermal regimes in the laboratory are presented in Chapter 5.

The use of wild populations in detecting differences between populations also raises questions about the effects of environmental conditions such as diet, season, age of the sample and temperature, all of which are known to affect cuticular CHCs. However, to solve this problem, by keeping other factors stable, an answer can be sought in future

studies by evaluating based on single variables. This study compared CHC profiles of adult flies of the same age with the rearing of first-generation eggs obtained from wild-collected adult flies to eliminate the age factor. In forensic entomology, more research is needed to establish how abiotic and biotic factors affect CHC profiles and how this can be linked to the crime scene. The data obtained in this study may allow estimating an insect's ability to adapt to changing climatic conditions.

Chapter 8

Summary, Conclusion and Future Research

8.1 Introduction

This chapter summarises the main outcomes of the studies undertaken and reported in this thesis. The summary of the results is divided into sub-headings according to the objectives that make up the chapters and salient findings. This is followed by a conclusion of the results and a presentation of their significance, along with recommendations for future research. Finally, novel aspects of the thesis are exposed.

8.2 Summary of Results

The main objectives of this thesis were to use the CHC profiles of two blowflies, *Lucilia sericata* and *Calliphora vicina*, reared outdoors by exposure to fluctuating temperatures, to identify the species and estimate the age of all developmental life stages. Additional objectives included evaluating the impact of temperature on CHC profiles in indoor and outdoor rearing, as well as identifying the variations between populations of the same species living in different habitats. The results given in Chapters 4 to 7 constitute the primary data for this thesis, which focuses on enhancing CHC analysis using GC-MS and applying the method in forensic entomology. The studies carried out comprised indoor and outdoor rearing experiments, combining three main aspects: CHC analysis, GC-MS and PCA.

In the first study of the thesis, presented in Chapter 4, the CHC profiles of *L. sericata* and *C. vicina* exposed to fluctuating temperatures in the field were compared and used for species identification for egg, larvae, pupae stages and empty puparia. By examining the effect of fluctuating temperature on the CHC profile of samples by rearing outdoors, the aim was to eliminate the limitations of previous studies carried out at a constant temperature.

The aim of the second study, presented in Chapter 5, was to examine the stability of the CHC profiles when two blowfly species were reared outdoors and to age the species through their life stages using the CHC analysis. It was also to determine the effect of temperature by comparing the CHC profiles of those reared outdoors exposed to fluctuating temperatures and indoors at a constant temperature.

The third study, expounded in Chapters 6 and 7, examined whether CHC profiles could be used to determine the difference between the populations of the same species collected from different habitats, namely the UK, Germany and Turkey.

8.2.1 Results of Species Identification Studies

In forensic entomology, the first step in calculating the PMI_{min} is to identify the species of entomological evidence, as different insect species have different life cycle timings. It is impossible to establish accurate ageing without the use of developmental growth data from the appropriate species. In this case, accurate species identification allows for a more precise determination of entomological data regarding development time [49]. CHC analysis has proven to have significant advantages over existing morphological techniques, as it can be performed for species identification at immature stage larvae, puparia, and empty puparia. The morphological techniques used to identify the species were explained under the headings of species identification in Chapter 4, as well as in sections 1.1.4 and 1.2.2.1. The results presented in Chapter 4 showed that species identification could be made for the blowfly eggs, first instar larvae, pupae and empty puparia of *L. sericata* and *C. vicina* species using CHC analysis combined with GC-MS and PCA. These results are characterised as species-specific differences due to being based on establishing differences by comparing species rather than identifying the species. Previous studies generally used stock generations that were reared in a laboratory

environment at a constant temperature for experimental investigation. However, the current research collected adult flies from the wild and their larvae were reared by exposure to fluctuating temperatures in the field. Thus, it was understood that the CHC profile is protected like a fingerprint despite the temperature factor, and CHC profiles can be used for taxonomy purposes regardless of environmental and temperature factors.

When a crime scene is investigated, it is critical to identify the egg species since it can provide information on the crime, but few studies have identified the egg stage by morphological characters. As in other life stages, DNA-based techniques can be used for species identification, but this is expensive and requires particular sequence knowledge [67]. Therefore, the CHC analysis technique was used to accomplish species-specific separation of *C. vicina* and *L. sericata* eggs. The results obtained are essential because of their contribution to the improvement of the CHC analysis.

Species identification of first instar larvae is usually difficult when undertaken by comparing the morphological characters. Therefore, they are reared until the post-feeding larvae stage, or adult fly stage, where morphological characters are larger in size and are thus easier to analyse. However, this is a time-consuming process [32]. With CHC analysis, species identification is possible in a short time and in a relatively easy way. With the findings of this study, it was seen that CHC profiles, which act as fingerprints, can be stably protected even the outdoors. Therefore, identification with the CHC analysis is possible.

The use of empty puparia left behind by adult flies after the pupae stage is rare for species identification because the characteristics of pupae species are very similar and the key standards published according to the species are limited. It was demonstrated in the results that the species identification of the pupae of *C. vicina* and *L. sericata* was performed on the first and last days of the pupae stage (the latter of which is the day just before the emergence of adult flies). The results further showed that the CHC profiles of the pupae could be used for species identification during the pupae stage. It was also found that even if pupae were reared under fluctuating temperatures, this did not interfere with identification. In addition, the empty puparia left behind by adult flies were successfully used to identify the species. The use of empty puparia for species identification may be problematic since adult flies shatter pupae when they hatch, making it difficult to

distinguish between species. Therefore, the results obtained are encouraging for the field of forensic entomology.

8.2.2 Results of Age Estimation Studies

One of the essential parts of determining PMI_{min} in a criminal investigation is age estimation. The oldest developmental stage discovered at the crime scene must be aged to estimate the time that has passed since colonisation, which is the only way to obtain accurate PMI_{min} estimation. In sections 1.1.5 and 1.2.2.2 and in Chapter 5, entomological evidence for age estimation using current techniques was explained. The results presented are essential in examining the effect of outdoor rearing on age estimation. Although fluctuating temperatures are often encountered at crime scenes, previous studies were carried out under constant temperatures in controlled laboratory conditions. Until this study, the effect of fluctuating temperature and outdoor rearing on CHC profiles and hence ageing was not known.

For daily ageing, studying morphological characters is difficult because instar stages can last longer than one day and quantifiable values, such as length and size, change with temperature and nutrition source [24]. However, it is possible to tell the stage of larvae by looking at posterior spiracles during the larval period. The ageing results obtained by CHC analysis for the larval stage were entirely satisfactory. The use of 3D PCA graphics was especially significant in terms of clustering the ageing samples day by day.

With morphological methods, it is difficult to determine the age of the pupae since the exterior characteristics of the puparial stage do not change for nearly the whole period. In addition, the chemical profile of the pupae is also difficult to age by CHC analysis, as the CHC composition of pupae is simpler. In order to overcome this difficulty, in this study, n-alkanes that are predominant both qualitatively and quantitatively but with minimal change were excluded from the PCA calculations, and the puparial stage was aged based on the changes in branched methyl alkanes and alkenes.

Previous longitudinal research over extended periods has shown that the CHC analysis technique can effectively age empty puparia [43]. Due to time constraints in the research stage, the ageing period was limited to a shorter period of 10 to 90 days. However, the

findings obtained, especially by comparing the experimental groups of *L. sericata* empty puparia buried in the field and maintained in the incubator, are significant in demonstrating the impact of environmental variables on the stability and ageing of the CHC profiles of the empty puparia.

Although the application of a single method for the age estimation of an adult fly seems successful with the studies carried out and the methods developed to date, it has been seen that only a rough idea can be obtained about the age of a sample and previous methods are far from being able to make age estimation with day-level accuracy. However, among the methods used, CHC analysis is currently the most promising and inexpensive method [160]. The results obtained in this thesis show that ageing, which is difficult to detect, such as the first five days for adult flies, can also be done in a short time. In addition, the results are encouraging for the improvement of the CHC analysis and its use in forensic entomology in indicating that age estimations can be made even if they are reared by exposure to fluctuating temperatures and revealing differences in the CHC profiles of rearing places.

8.2.3 Results of Population Comparison Studies

In forensic investigations, determining the nature of the crime scene is critical. This can include determining whether a victim died or had been killed at the crime scene or was killed elsewhere and was subsequently transferred to the site of the corpse discovered. Entomological evidence may help identify the crime scene and determine whether the corpse has been relocated [166]. The results presented in section 1.2.2.3 and in Chapters 6 and 7 reveal inter-population differences between samples of the same species living in different habitats. In Chapter 6, the differences in CHC profiles between eggs, larvae and pupae of *C. vicina* and *L. sericata* populations collected and reared outdoors in the UK and Germany were comparatively investigated. In Chapter 7, in addition to these two populations, the Turkish population was added, and the differences in CHC profiles between adult flies and their empty puparia were examined. These studies used first-generation eggs of *L. sericata* and *C. vicina* adult flies collected from the UK, Germany and Turkey. Most previous research used wild-collected adult flies maintained in laboratories, but this study used blowfly eggs of wild female adult flies reared in outdoor

cages. The temperature was recorded using an individual datalogger to examine the temperature effect on the CHC profiles. The main goal was to compare populations rather than to find particular chemicals. The detection or non-detection of chemicals in CHC profiles and their quantitative values were utilised to identify inter-population differences. The same component may be found in both profiles, but differing ecological and physiological features may induce quantitative composition. Qualitative and quantitative data helped identify populations.

The distance between the UK and Germany is relatively short, and their climates are generally comparable. On the other hand, Turkey is geographically separated from these two nations and has a climate that is distinct from them. Turkish weather is warmer and dryer, whereas Germany and particularly the UK are cooler and more humid. This was evident in the studies conducted on the life cycle stages of the *C. vicina* and *L. sericata* species. The CHC compounds with a long carbon chain and a higher boiling point were found abundant in the chemical profiles of the Turkish population. By comparing the chemical profiles of the German and UK populations, it can be concluded that the populations of these two nations reflect the same environmental features. The results presented hold great promise for the identification of species from different geographical locations. Because these variations were found in the CHC profiles, it is possible that there are variances in chemical composition between the two species as a result of climatic differences.

8.3 Conclusion

This study successfully explored and discussed species identification, ageing and inter-population differences for *C. vicina* and *L. sericata* life cycle stages collected from the UK, Germany and Turkey and reared in the field. The impact of indoor and outdoor rearing on CHC profiles was also shown. The chemical identification of CHCs was accomplished via the use of the GC-MS analytical method in conjunction with a mix of PCA and statistical analysis. The fact that the CHC analysis gives results in such a broad sense in a short time is very advantageous in terms of compensating for the time consumed during forensic investigations.

The following findings were obtained as a consequence of the comprehensive research that was carried out and described in this thesis:

- *C. vicina* and *L. sericata* colonies were established from the blowfly eggs of adult flies collected from the wild and were reared in field-placed cages. Temperatures were recorded with an individual datalogger. This study demonstrates the utility and stability of CHC profiles obtained by extracting blowfly eggs, larvae, puparia, adult flies and their empty puparia exposed to fluctuating temperatures in species identification and age estimation.
- The effect of constant and fluctuating temperatures on the stability of the CHC profiles was investigated by comparing the CHC profiles of larvae, puparia, adult flies and their empty puparia exposed to a fluctuating temperature in the field (outdoors) and at constant temperatures in an incubator under standard laboratory conditions (indoors). Thus, the effects of temperature on CHC profiles were thus demonstrated.
- The research was conducted to determine the effects of indoor and outdoor conditions on the CHC profiles of *L. sericata* empty puparia that had been buried and placed in a constant temperature incubator as a control group. This study showed the effects of weather conditions on the chemical profile of empty puparia.
- The chemical profiles of eggs, larvae, and pupae stages obtained from adult flies of *C. vicina* and *L. sericata* collected from the UK and Germany were examined to detect inter-population differences. Since the experimental sets were placed and reared in the fields, the temperatures they were exposed to during the developmental periods were recorded. Thus, the effect of the ambient temperature on the CHC profiles was revealed.
- In order to determine the geographical differences in the chemical profiles of the same species living in different habitats, first-generation adult flies obtained from adult flies of *C. vicina* and *L. sericata* were collected from the wild in the summer months in the UK, Germany and Turkey, and their empty puparia were investigated. Thus, environmental differences between populations and cross-country climate changes were shown to affect CHC profiles.

- CHC analysis is straightforward; its findings may be acquired in a short time, and its extraction methods can be followed and performed by non-expert practitioners. Compared to advanced methods such as DNA-based approaches, GC-MS analysis is less costly and takes less time to complete. It is possible to differentiate between CHC profiles using extracts from each sample and the chromatogram produced via GC-MS analysis. In order to understand and visualise the obtained chromatogram data, it may be applied to a PCA model that provides information about the insect, such as its species or age.

8.4 Suggestions for Future Research

This study has made significant new contributions to the area of forensic entomology. Through a series of experiments described in the thesis, it has been possible to demonstrate many uses of CHC analysis. Nevertheless, there remains scope for future research to demonstrate the capability of the method further.

It is necessary to conduct research that evaluates the validity and applicability of CHC analysis in forensic investigations. It remains unclear what procedure should be followed to extract CHC profiles of entomological evidence directly from a crime scene. The effects of on-scene extraction of chemical profiles required for CHC analysis or preservation of an insect sample in solution on CHC profiles are still unknown.

It is important first to create a database of CHC profiles for forensically important species. It is essential to advance the CHC analysis technique to establish a database, with research being conducted over a broader range of locations and with additional species being included. The primary aim of this database would be to load a CHC profile and determine whether or not the profile matches a species. This thesis investigated the impact of rearing circumstances and regional variations on their chemical profiles using two species from three different populations, and they were reared in their native climates. In future research, if the species obtained from various populations are reared at a constant temperature to eliminate the temperature effect, the differences between populations may be addressed more directly. Although species have fingerprint-like CHC profiles, differences in relative proportions have been identified between populations. However, since only a limited number of populations of *C. vicina* and *L. sericata* species were

examined, it is not known whether the results obtained are valid for this study or for all populations in general, which will form the main subject of future studies.

It is easier to distinguish populations from each other with CHC analysis than with methods using morphological characters. Nowadays, GC-MS is an integral part of almost all laboratories, and CHC analysis is a relatively inexpensive and easy method in forensic entomology, with obvious utility in criminal investigations. Moreover, distinguishing geographical populations by analysis of CHC profiles has the potential to be a valuable forensic tool for future studies.

PCA was selected as the statistical approach for testing the validation of the CHC method for both indoor and outdoor samples in the present experiments since it is basic, common and accessible. Further research into the effects of indoor and outdoor rearing on ageing will be beneficial in the future. More comprehensive methods such as artificial neural network analysis [42,148], which can work with more data efficiently, can be used to examine the effect of indoor and outdoor spaces on ageing in greater depth.

Many studies in the literature examine the temperature effect on CHC profiles of fruit flies; however, few studies show the effect on blowfly species. In future studies, it is essential to examine the effects of different temperatures and temperature shifts on the CHC profiles of forensically important species to eliminate the limitations in this area.

It is also unknown how the victim's medication history or drug usage affects the chemical profile of the larvae. Studies that will be carried out in this area may prove beneficial in the field of entomotoxicology.

Aside from the primary applications of CHC analysis in forensic entomology, such as species identification and age estimation, the application of CHC analysis to museum samples will also be of considerable use to catalogue collections.

8.5 Research Contributions

The experimental work carried out for this thesis has yielded essential knowledge that will be of use to researchers and forensic practitioners in the future. The following are the predominant contributions of this thesis:

- Outdoor experiments conducted in the field highlighted the remarkable utility of CHC analysis for crime scene investigations. In forensic entomology, experimental data obtained in previous studies were obtained under standard laboratory conditions, but certain field-based applications were required to thoroughly verify the CHC analysis and assess the viability of the method. Therefore, in this thesis, a series of studies covering the ambient environment and standard laboratory conditions were conducted to examine the stability of CHCs at constant and fluctuating temperatures and to determine their impacts on chemical profiles.
- As a result of this investigation, it has been shown that species have CHC profiles like a fingerprint. Furthermore, it has been shown that various populations of the same species might indicate that there may be variances between CHC profiles; this holds great promise for the development of a more accurate method of CHC analysis.

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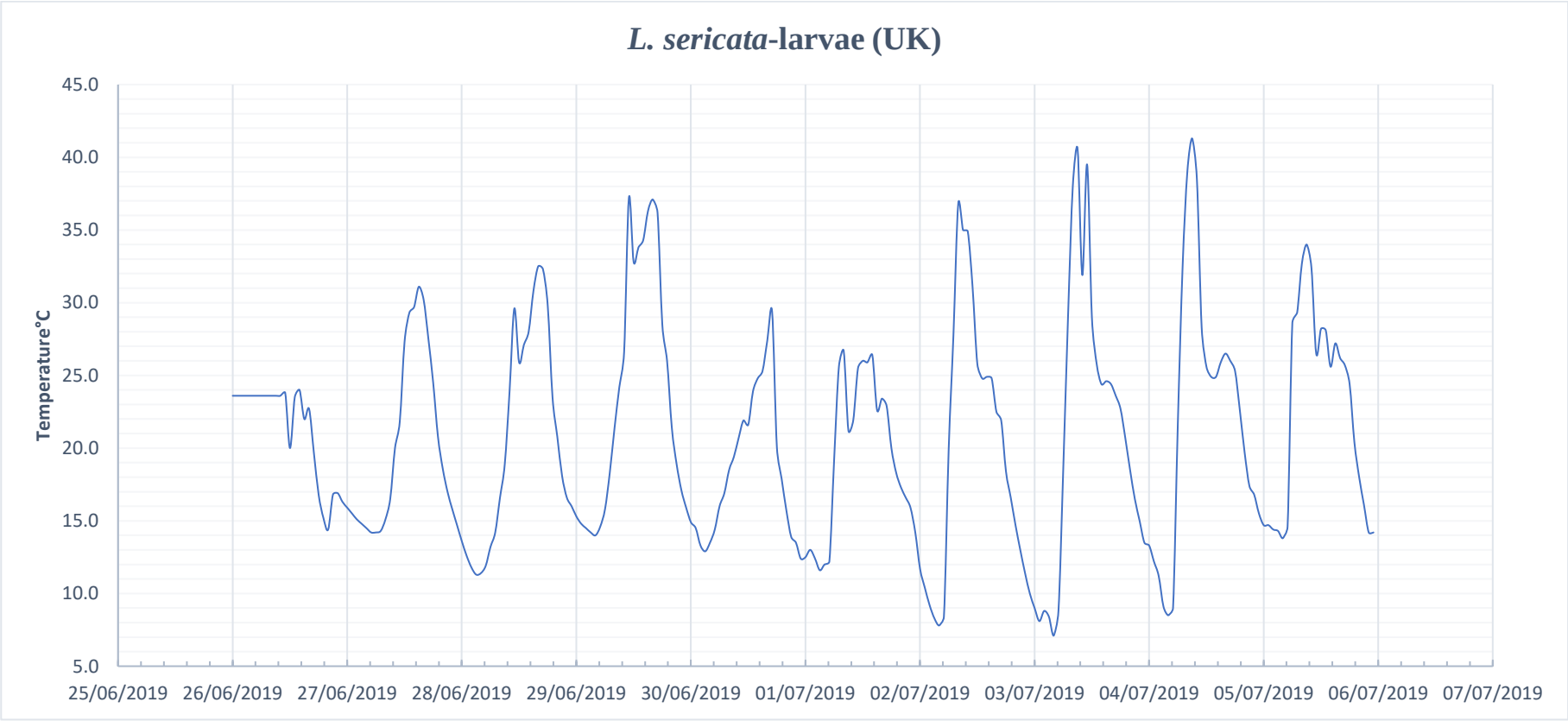
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Appendix 1: Temperatures (Swindon (UK)) during *L. sericata* larval period

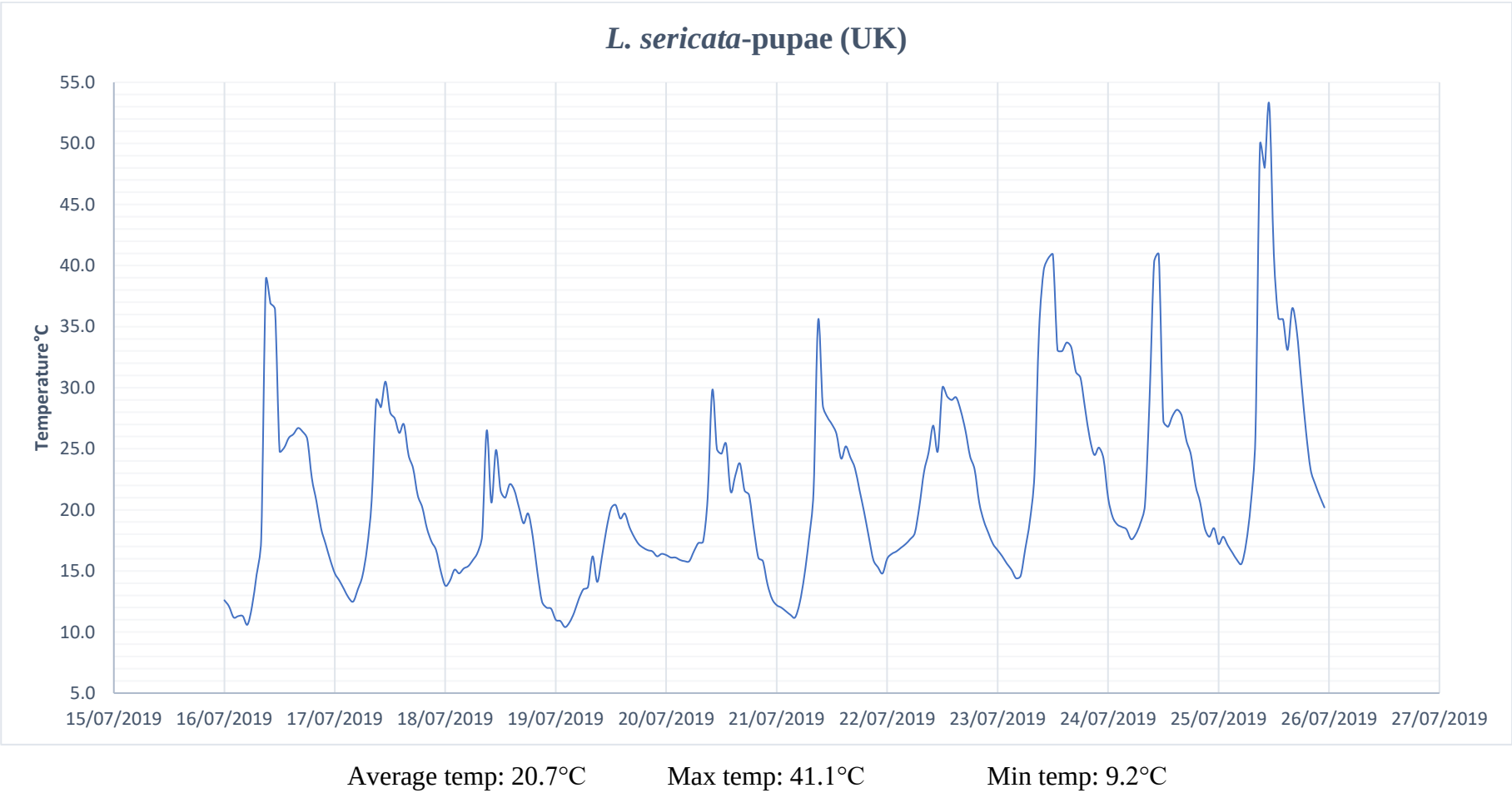


Average temp: 20.9°C

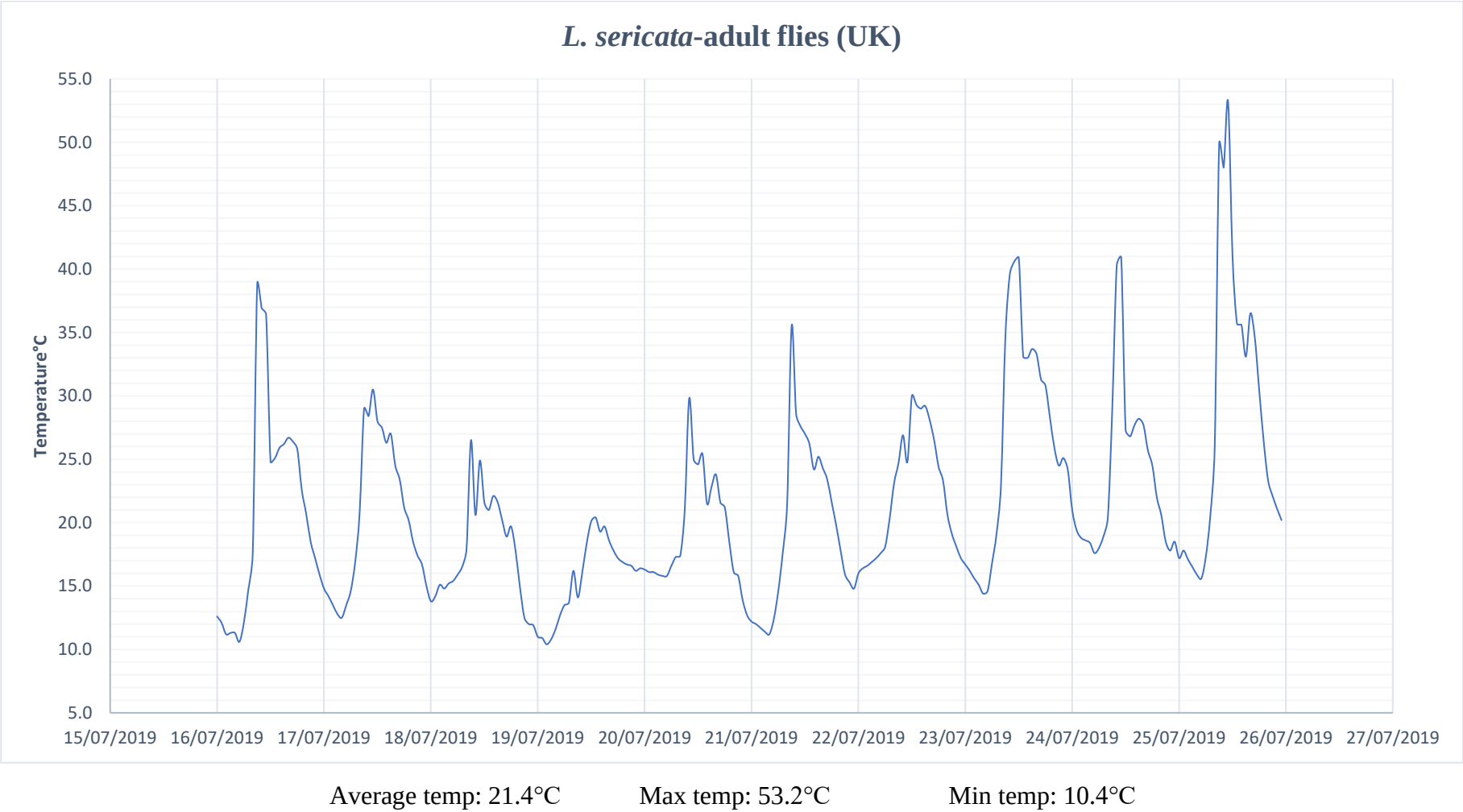
Max temp: 41.3°C

Min temp: 7.1°C

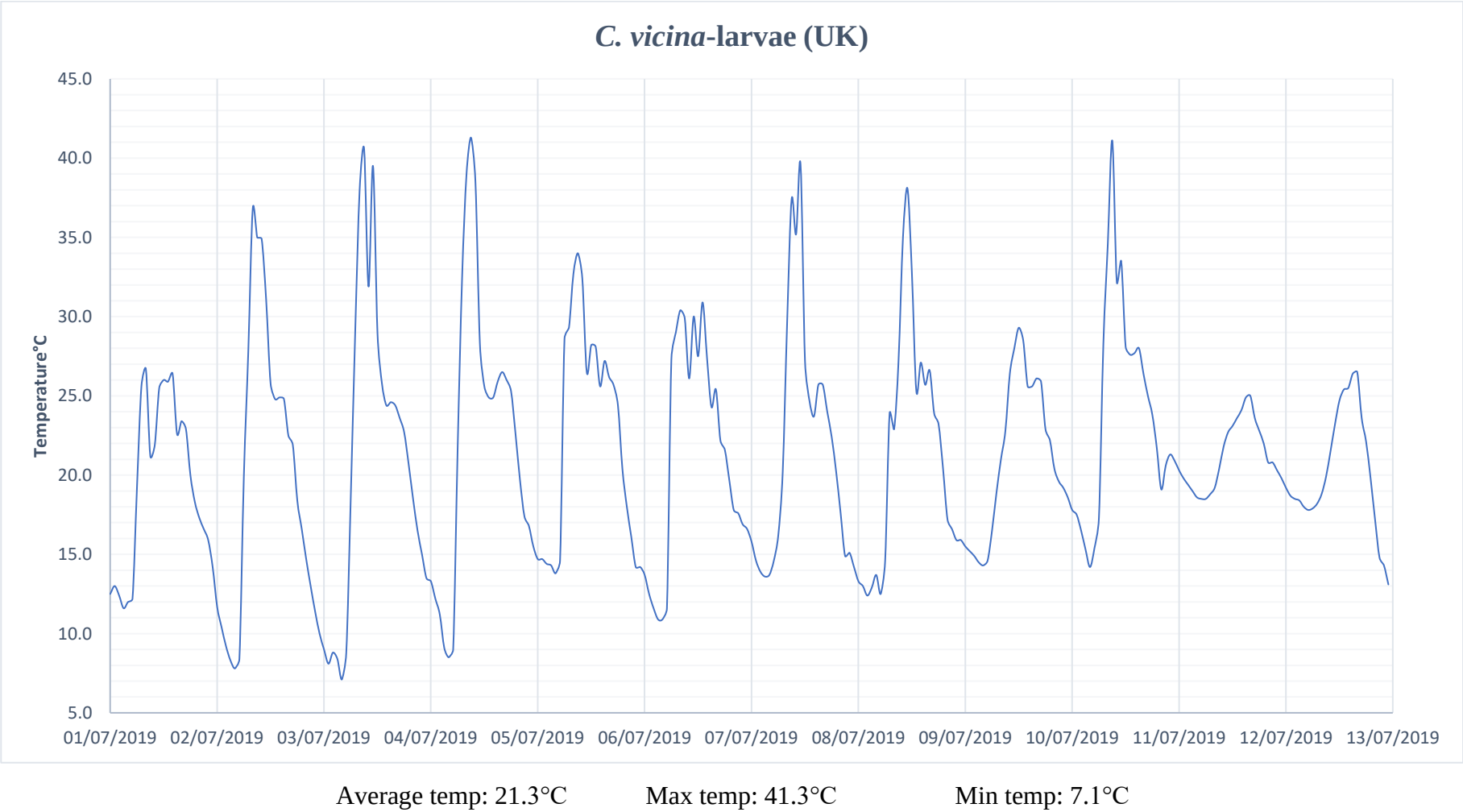
Appendix 2: Temperatures (Swindon (UK)) during *L. sericata* puparial period



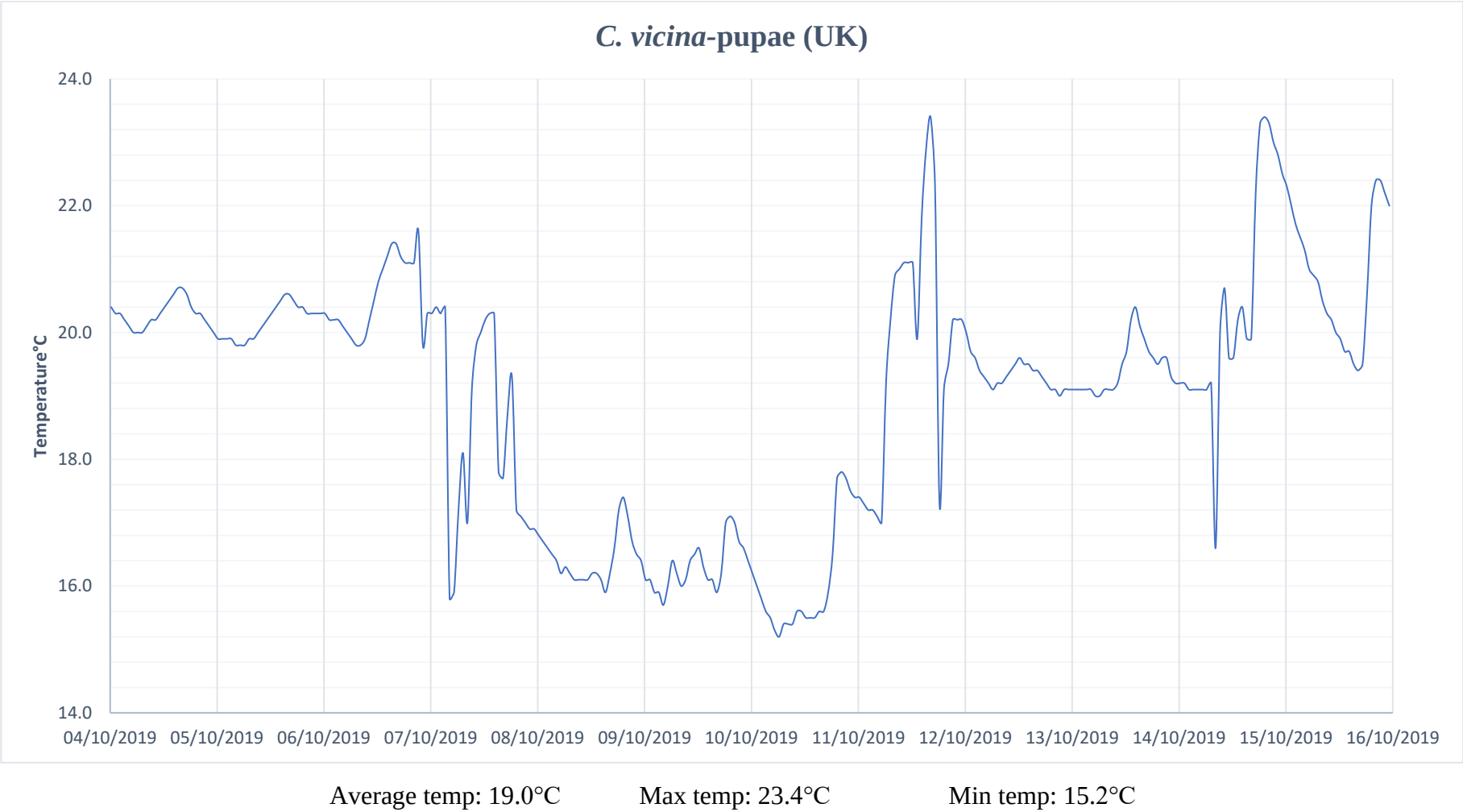
Appendix 3: Temperatures (Swindon (UK)) during *L. sericata* adult fly period



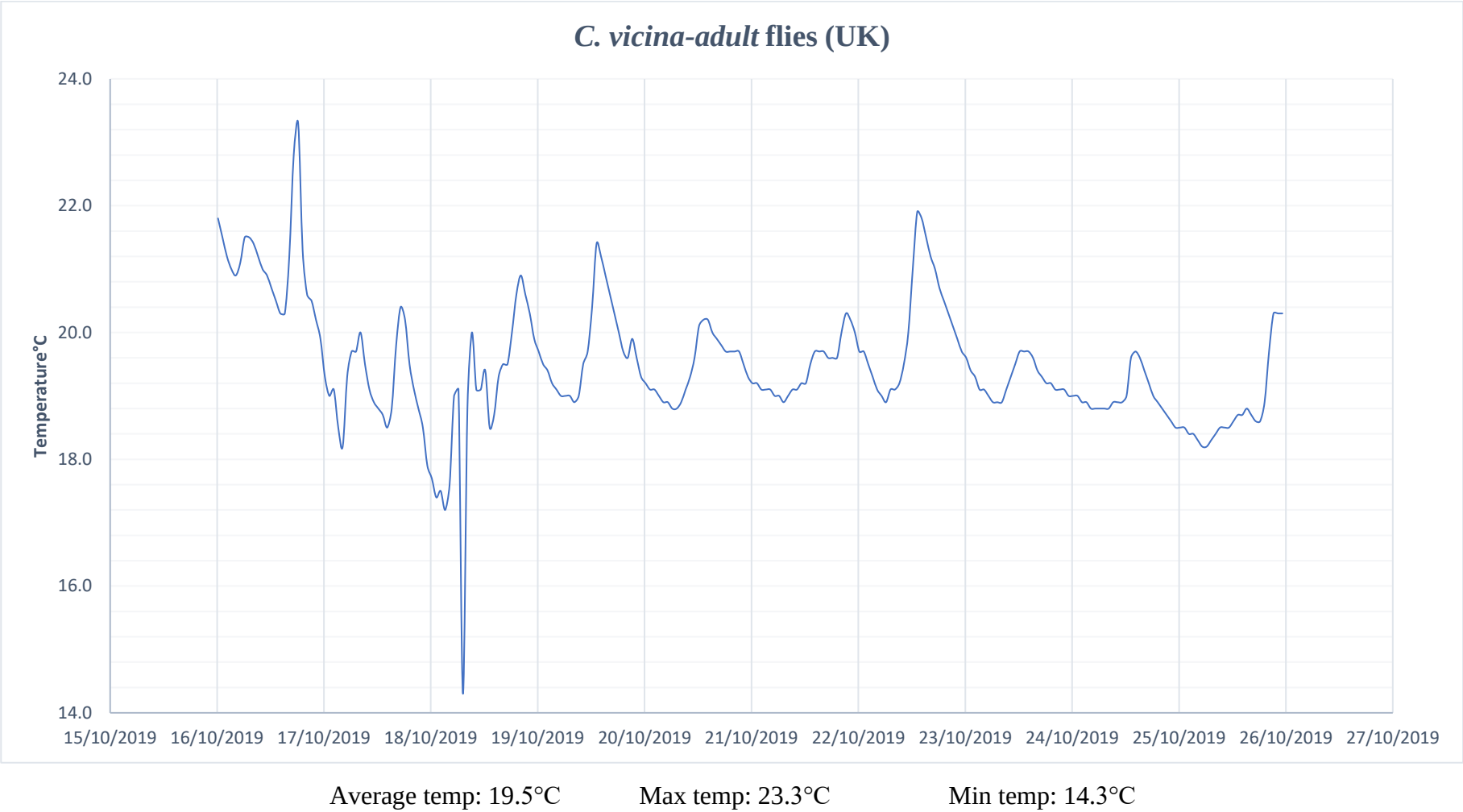
Appendix 4: Temperatures (Swindon (UK)) during *C. vicina* larval period



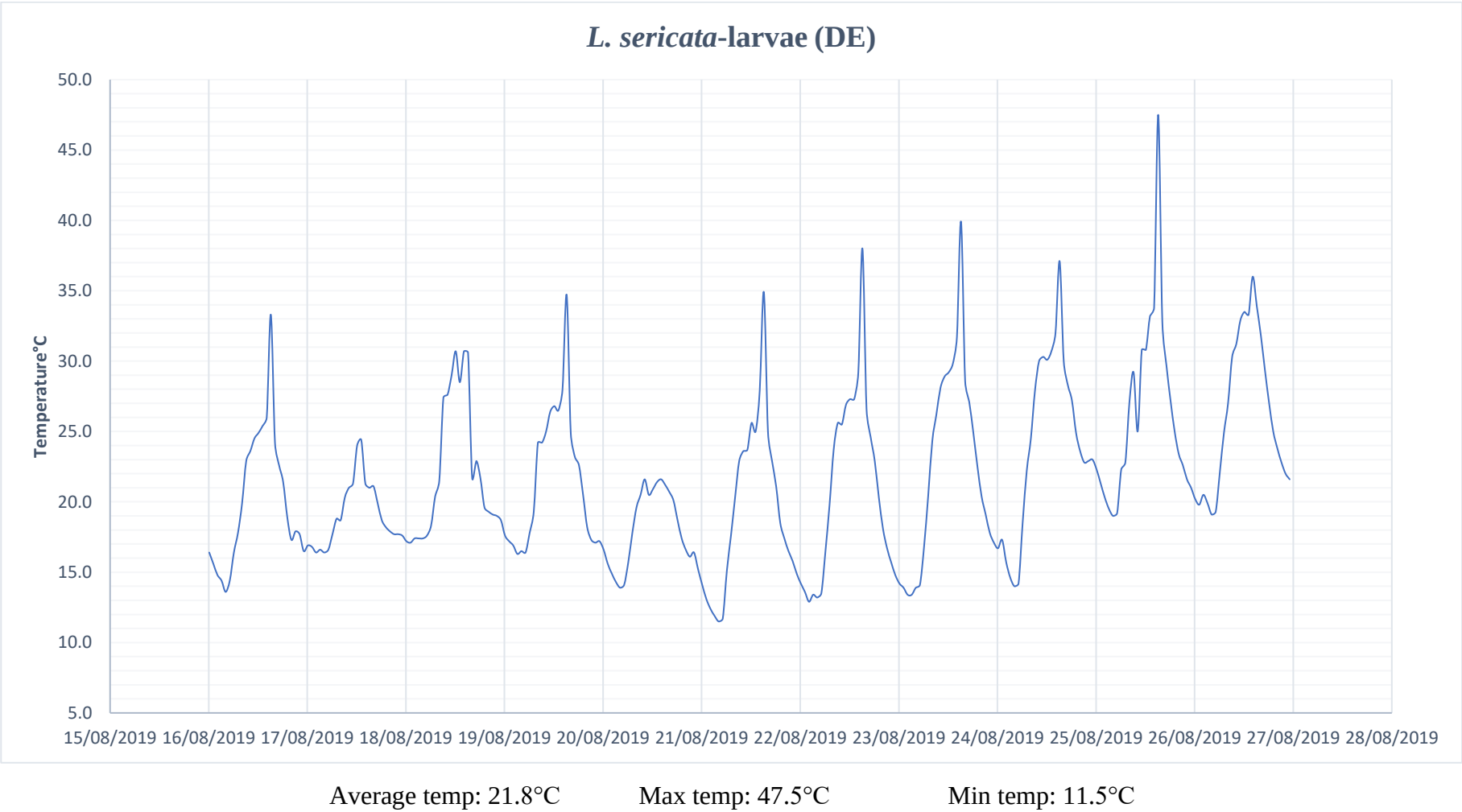
Appendix 5: Temperatures (Swindon (UK)) during *C. vicina* puparial period



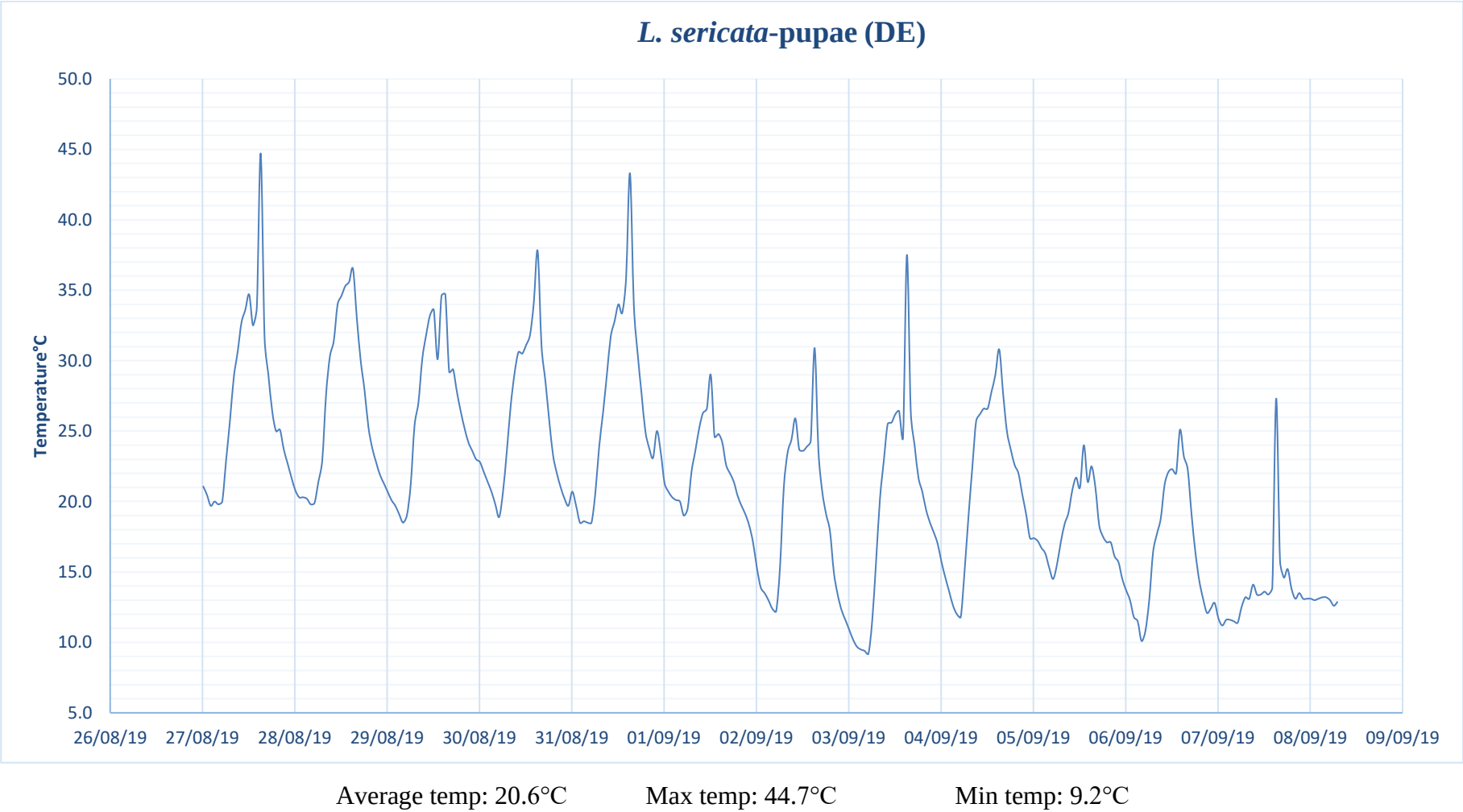
Appendix 6: Temperatures (Swindon (UK)) during *C. vicina* adult fly period



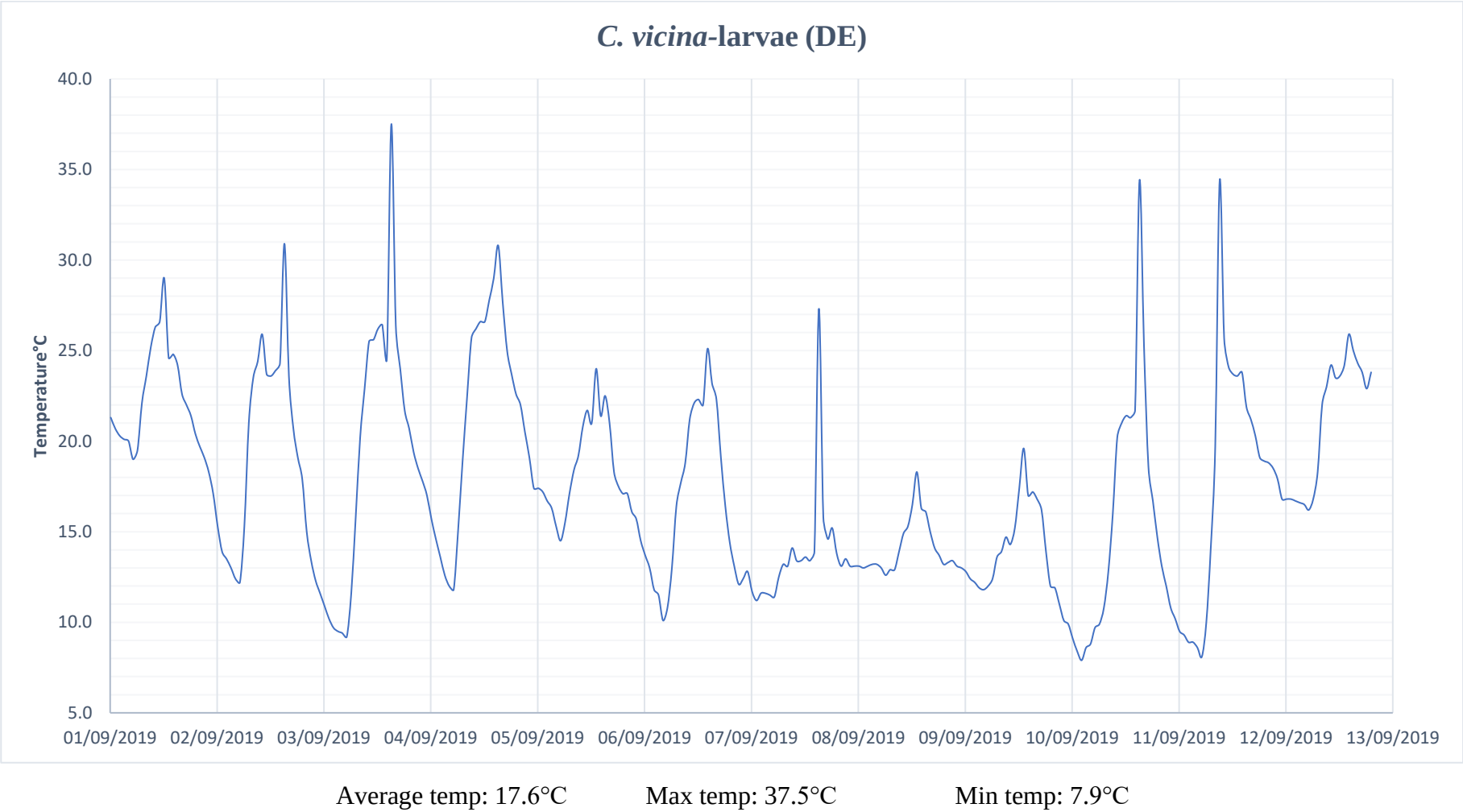
Appendix 7: Temperatures (Frankfurt (Germany)) during *L. sericata* larval period



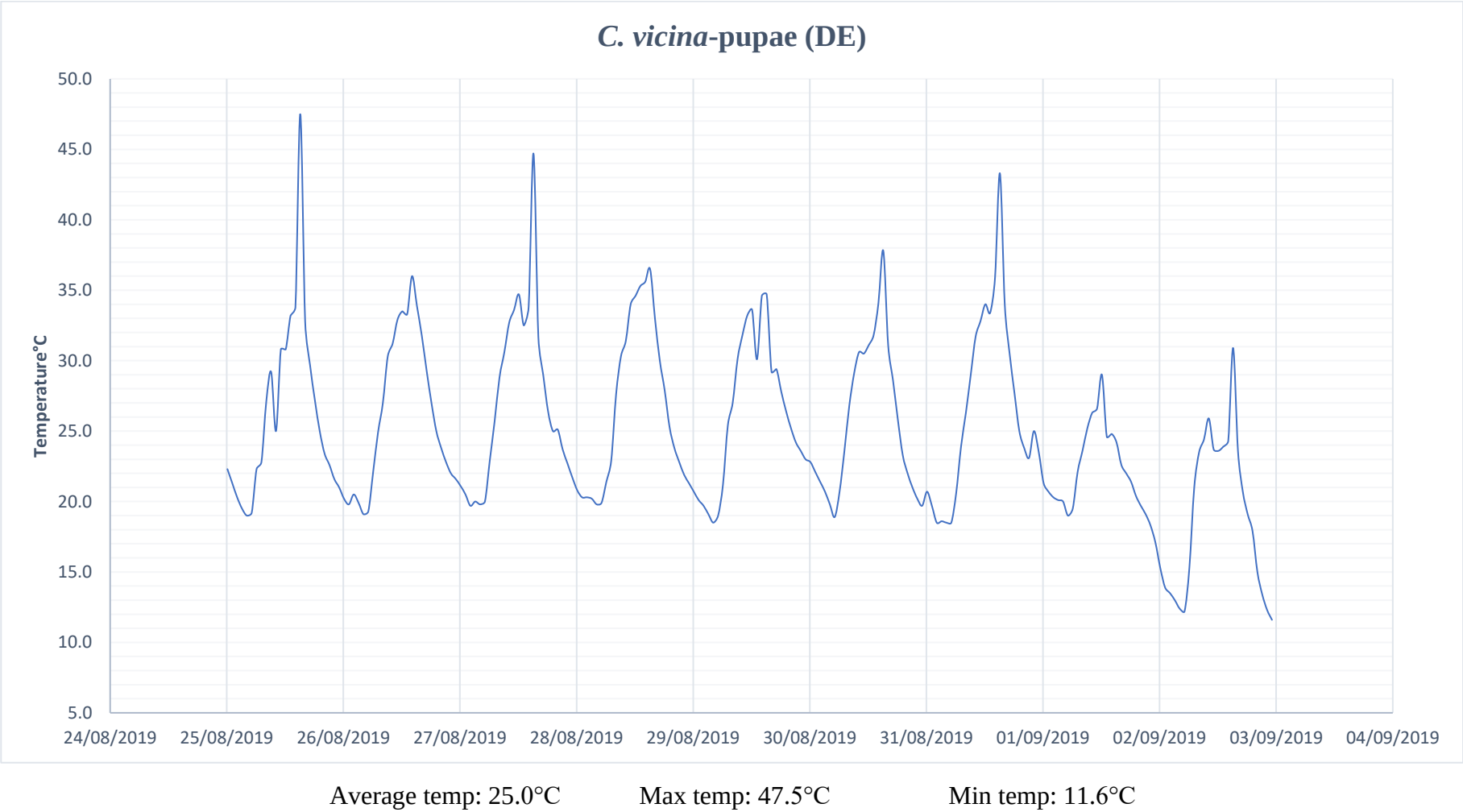
Appendix 8: Temperatures (Frankfurt (Germany)) during *L. sericata* puparial period



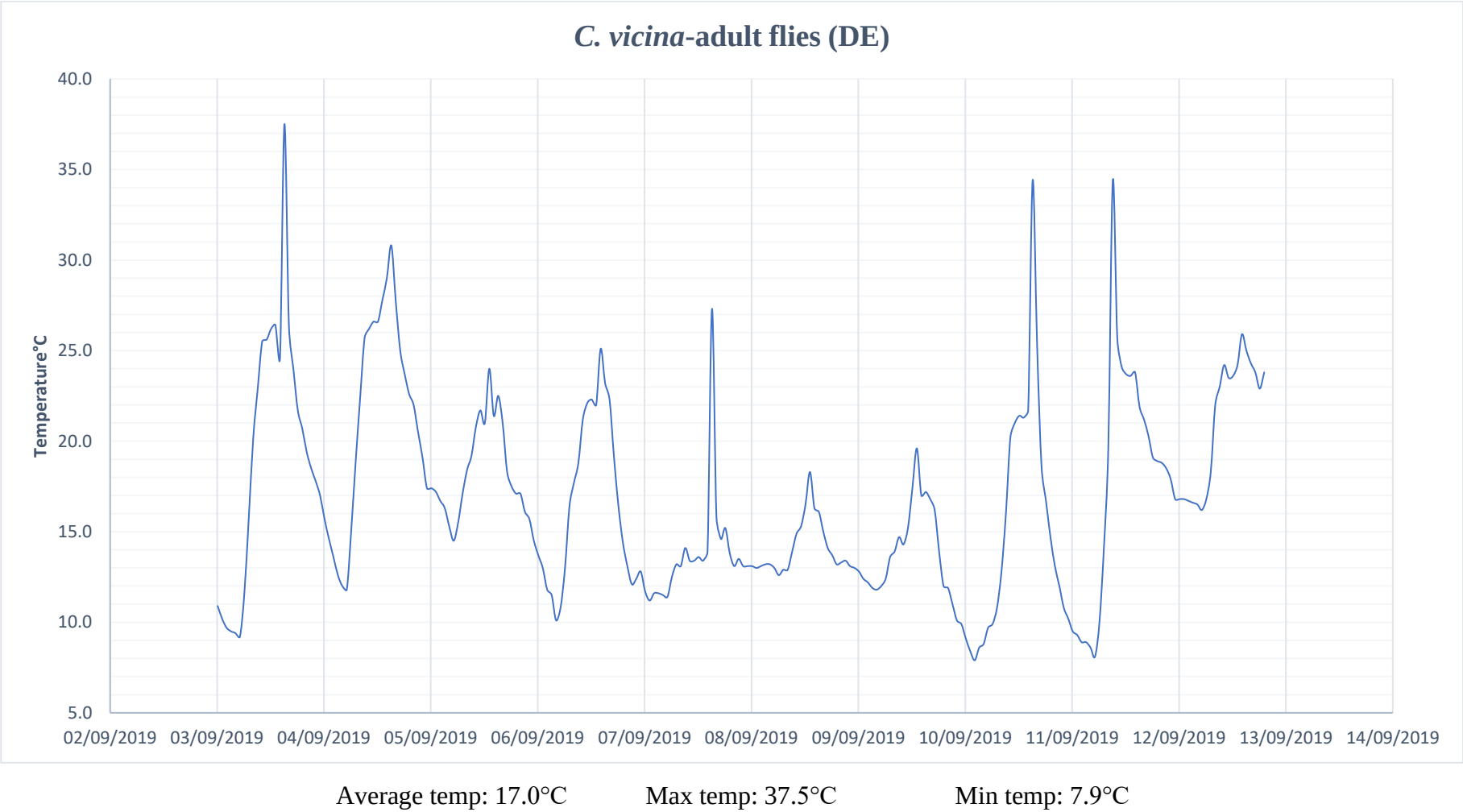
Appendix 9: Temperatures (Frankfurt (Germany)) during *C. vicina* larval period



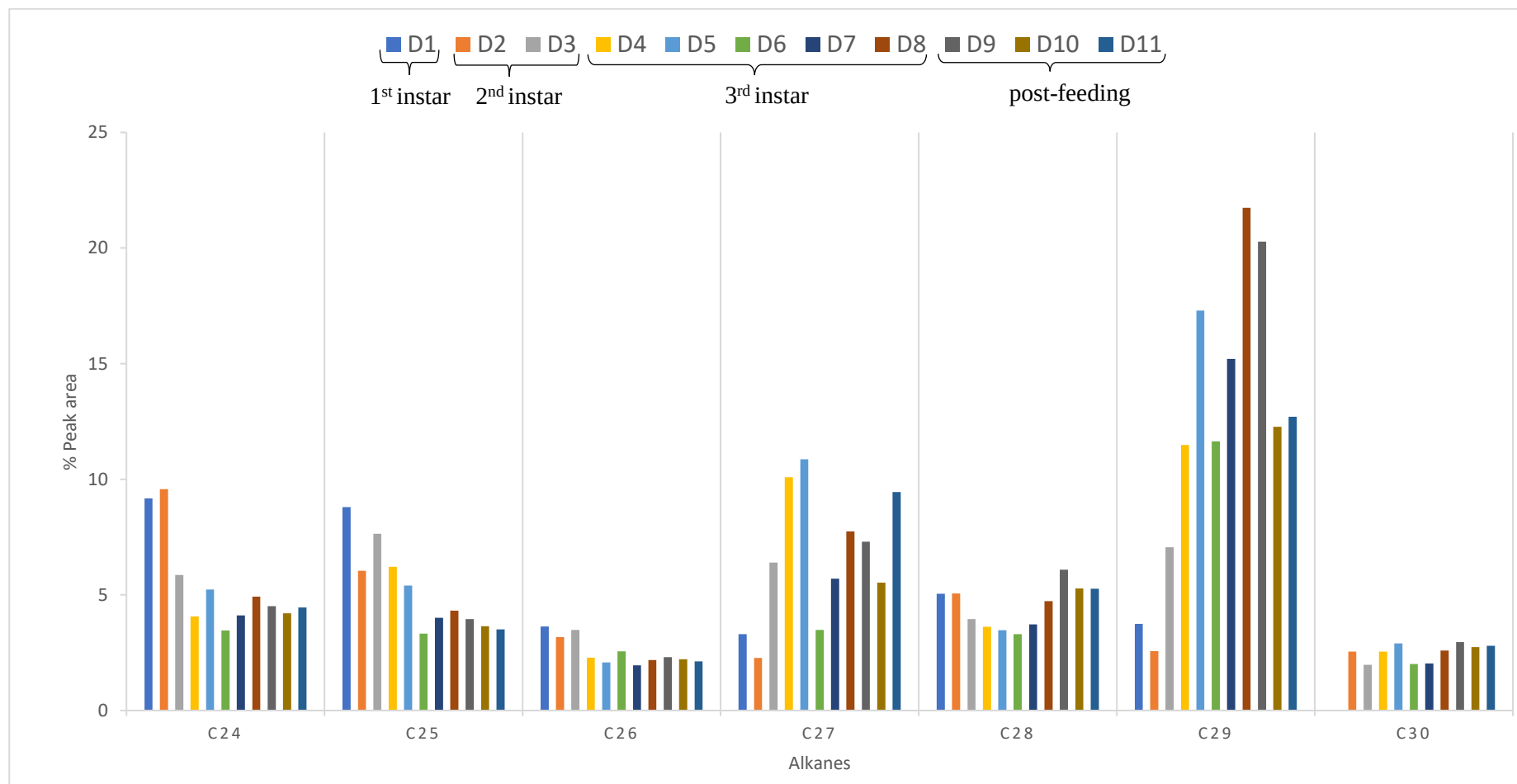
Appendix 10: Temperatures (Frankfurt (Germany)) during *C. vicina* puparial period



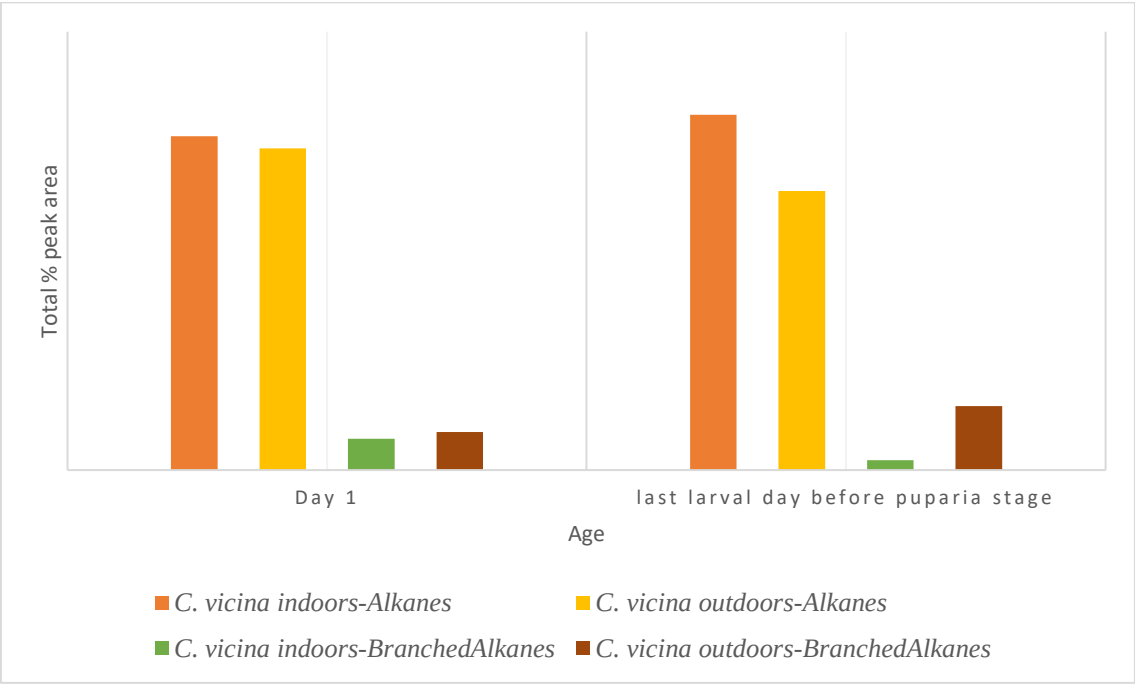
Appendix 11: Temperatures (Frankfurt (Germany)) during *C. vicina* adult fly period



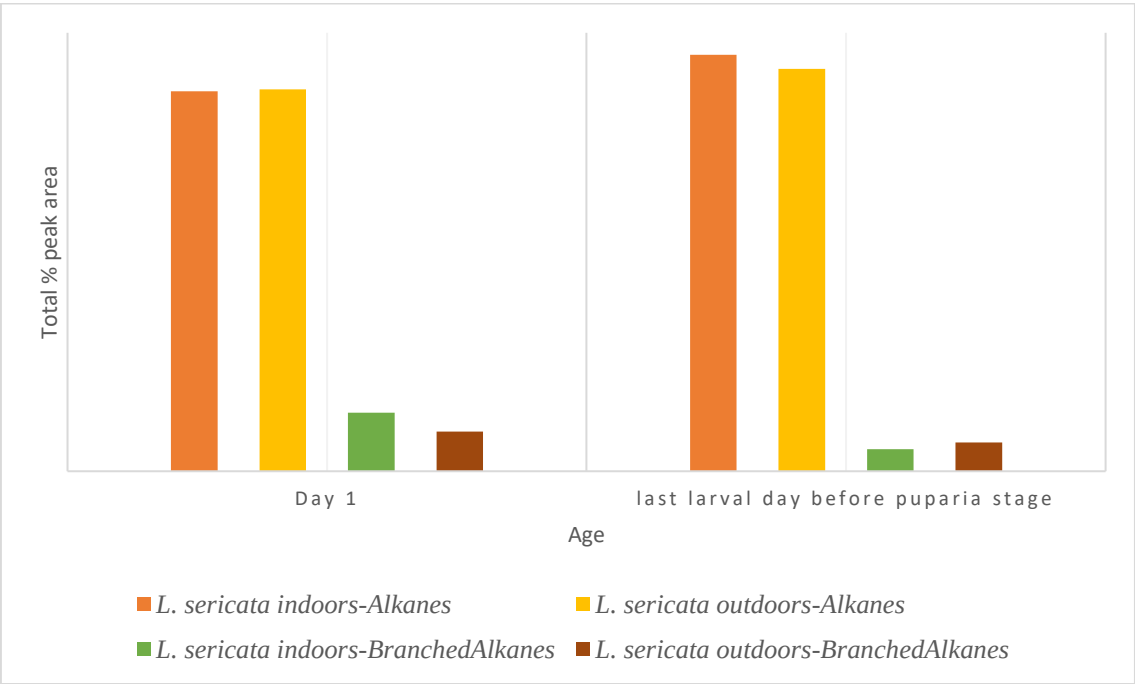
Appendix 12: Percentage concentrations of C24:H to C30:H n-alkanes over larval period of *C. vicina* reared outdoors in UK



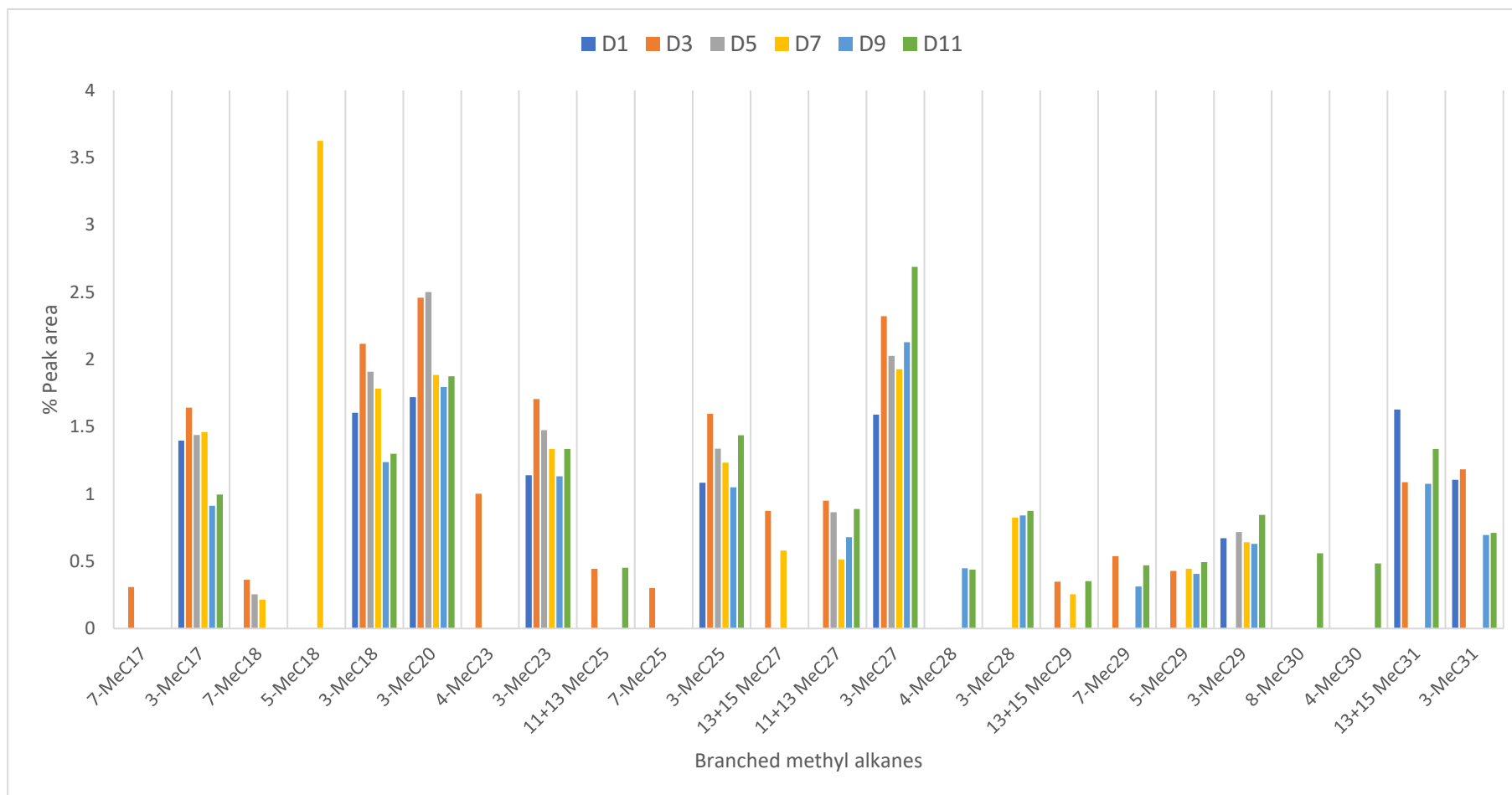
Appendix 13: Total percentage concentration of n-alkanes and branched methyl alkanes for Day 1 and last day of *C. vicina* larvae reared outdoors and indoors



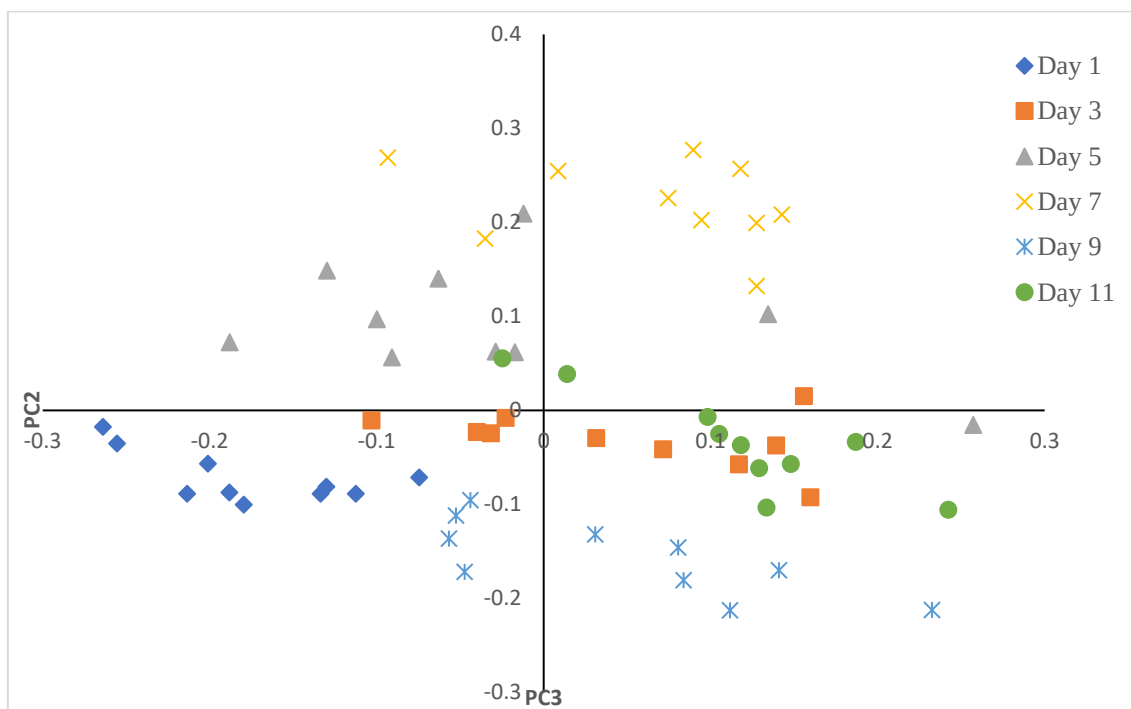
Appendix 14: Total percentage concentration of n-alkanes and branched methyl alkanes for Day 1 and last day of larvae of *L. sericata* reared outdoors and indoors



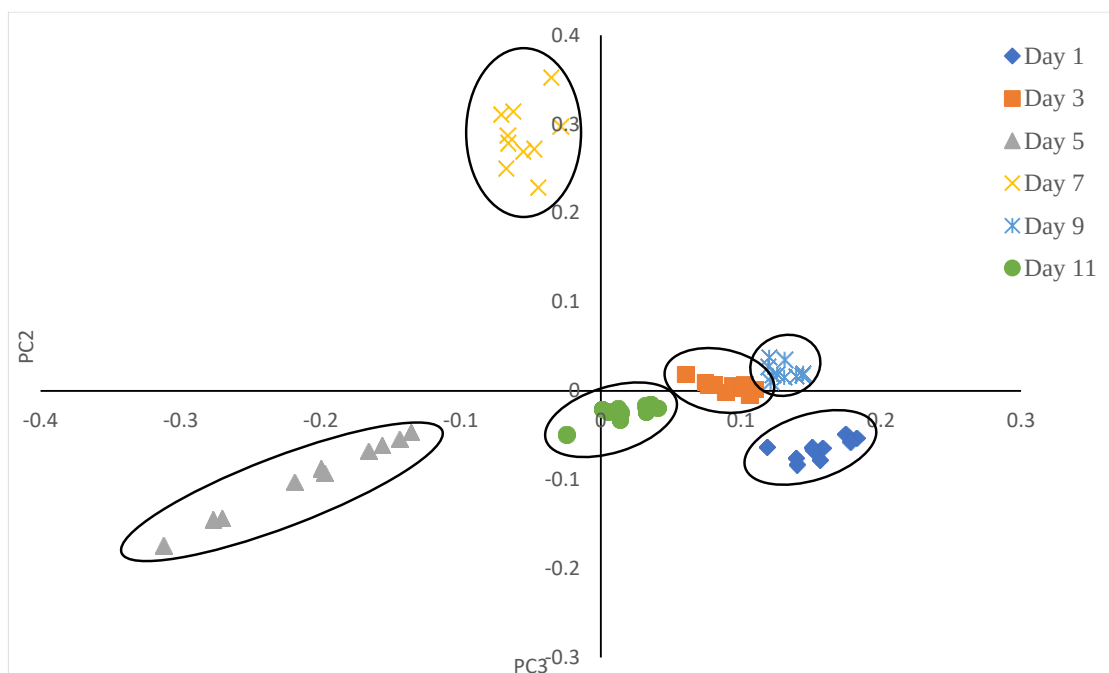
Appendix 15: Percentage concentration of branched methyl alkenes of *C. vicina* puparia pupae reared outdoors in the UK during the puparial period



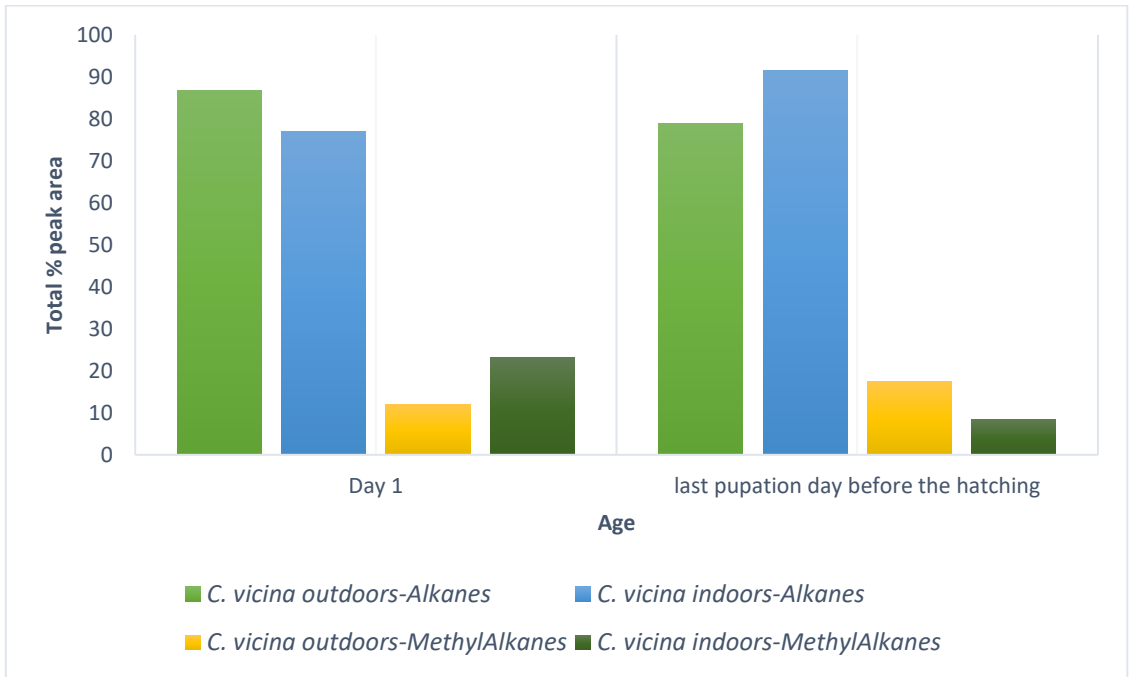
Appendix 16: PCA plot showing PC3 against PC2 for *C. vicina* pupae reared outdoors, using n-alkanes, alkenes and branched methyl alkanes



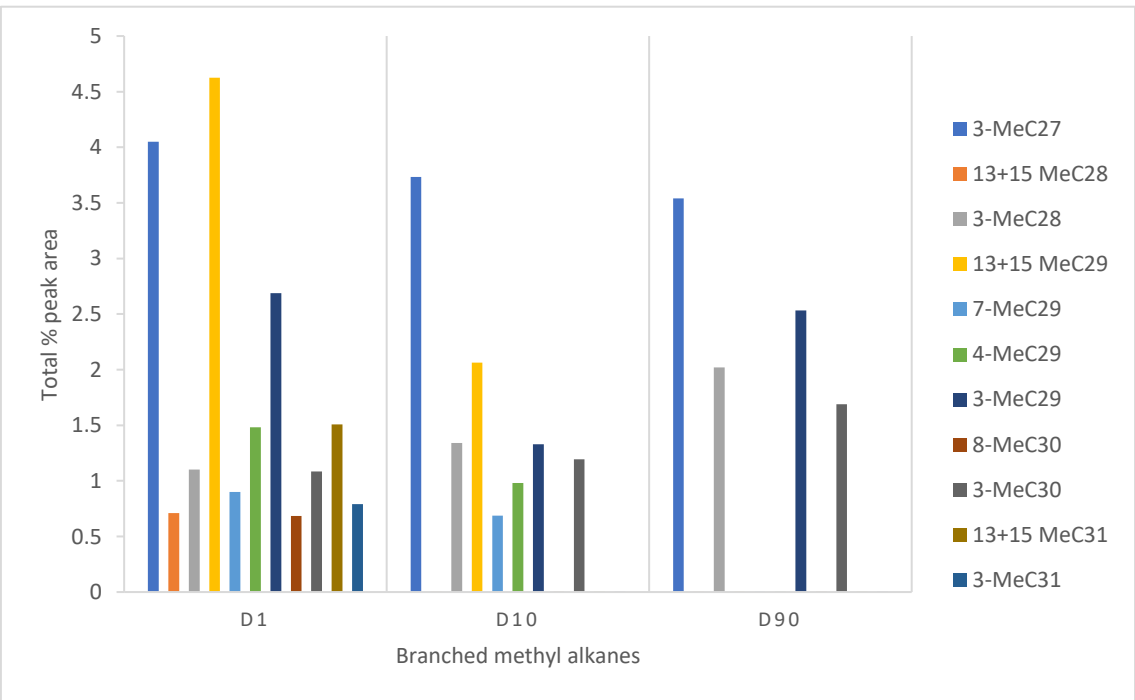
Appendix 17: PCA plot showing PC3 against PC2 for *C. vicina* pupae reared outdoors, using alkenes and branched methyl alkanes only, with clustering days circled



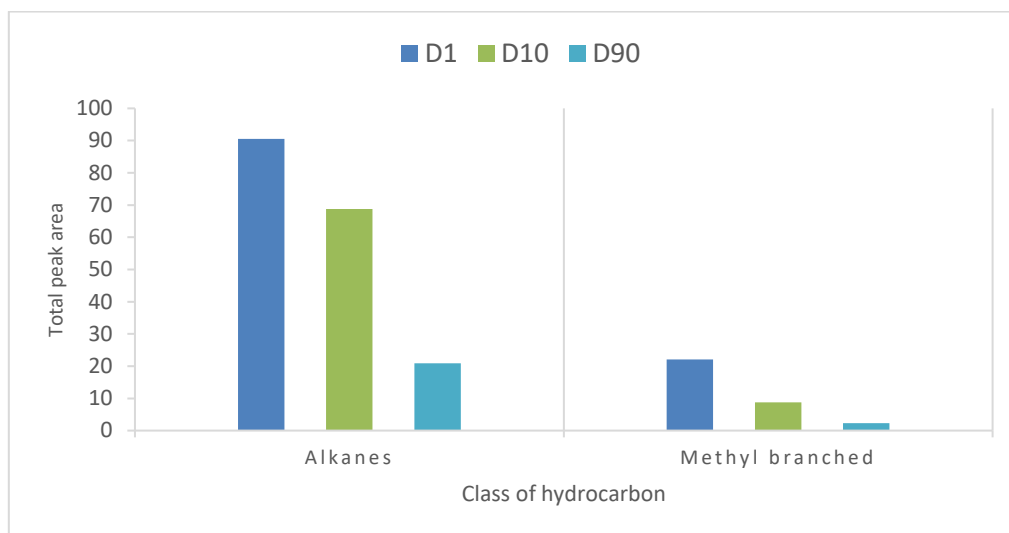
Appendix 18: Total percentage concentration of n-alkanes and branched methyl alkanes for Day 1 of pupation and last pupation day before eclosion of *C. vicina* reared outdoors and indoors



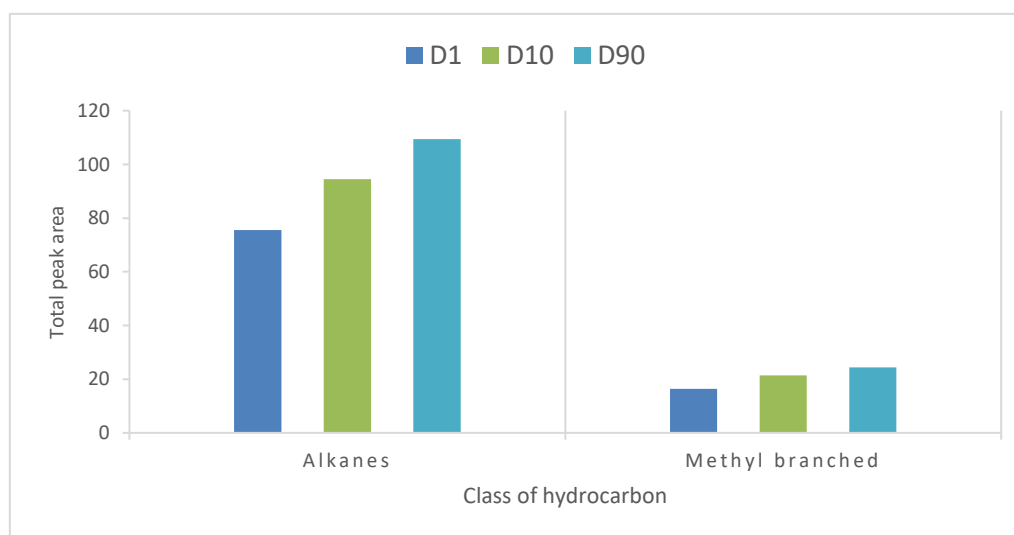
Appendix 19: Plot showing the total concentrations of branched methyl alkanes present over the empty puparia for *L. sericata* stored outdoors for 90 days



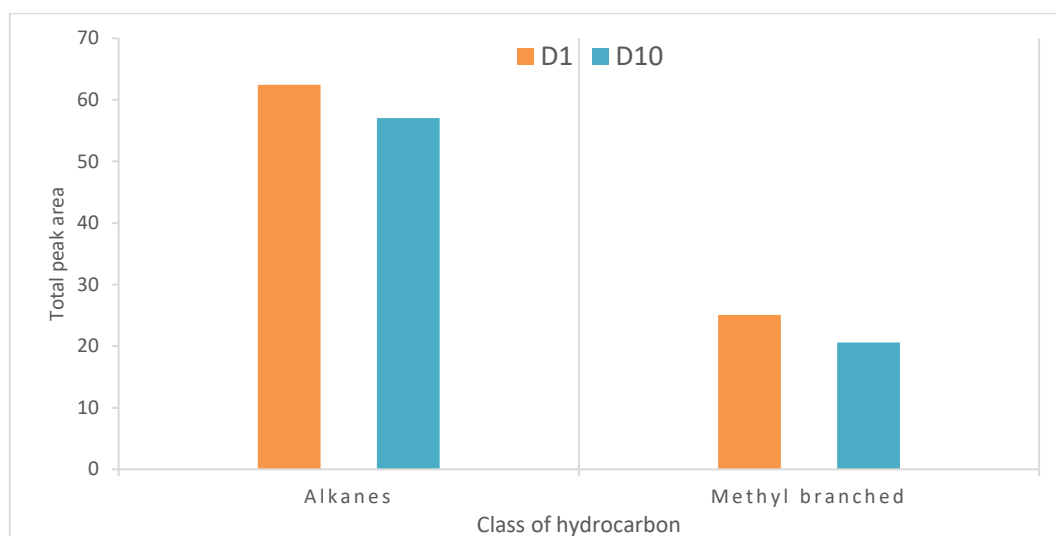
Appendix 20: Plot showing the total concentrations of n-alkanes and branched methyl alkanes present over the empty puparia of *L. sericata* stored outdoors for 90 days



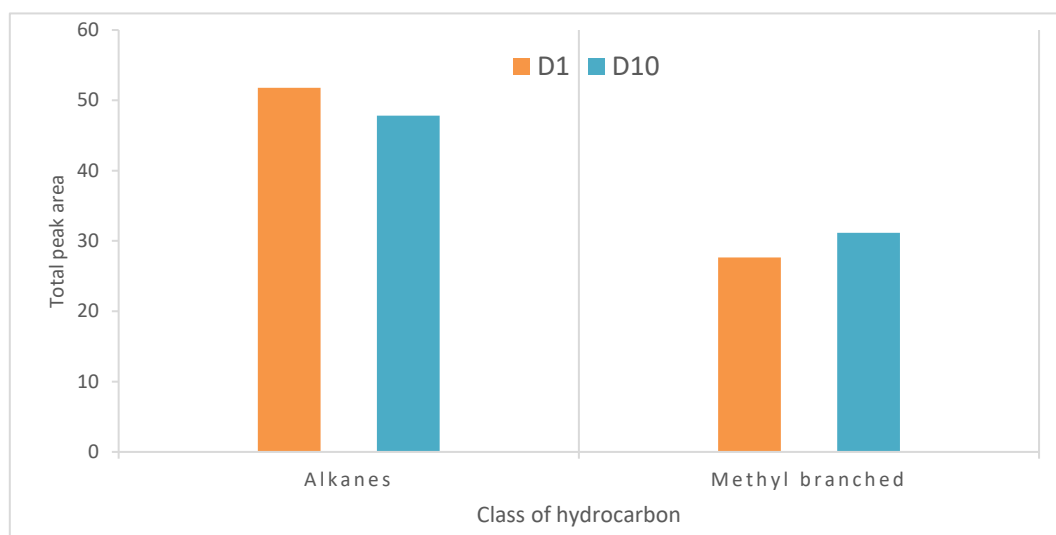
Appendix 21: Plot showing the total concentrations of n-alkanes and branched methyl alkanes present over the empty puparia for *L. sericata* stored indoors for 90 days



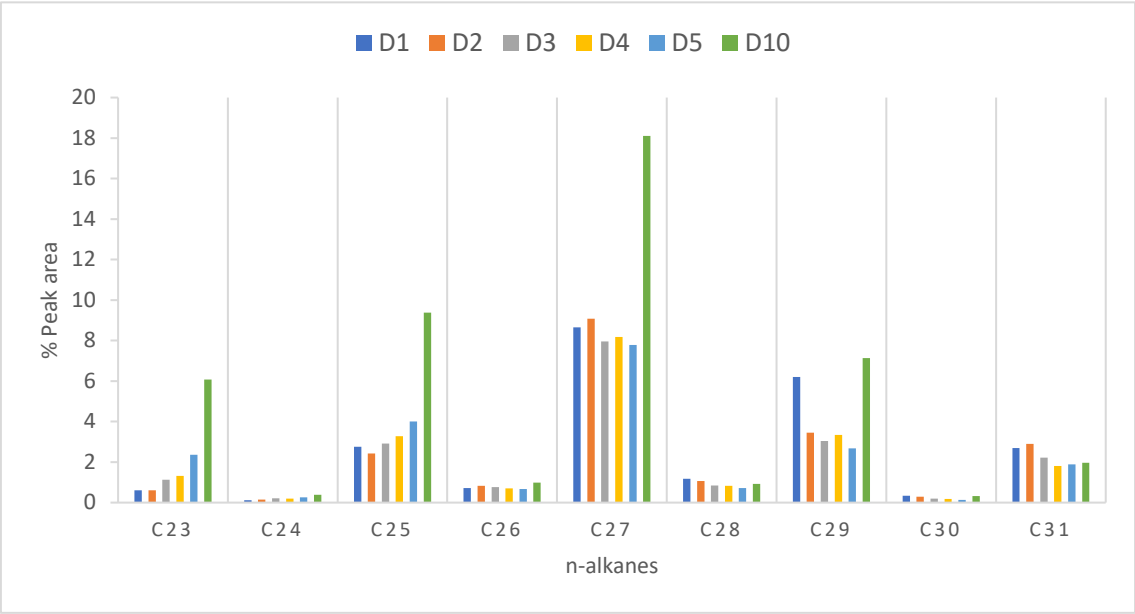
Appendix 22: Plot showing the total concentrations of n-alkanes and branched methyl alkanes present over the empty puparia for *C. vicina* stored outdoors



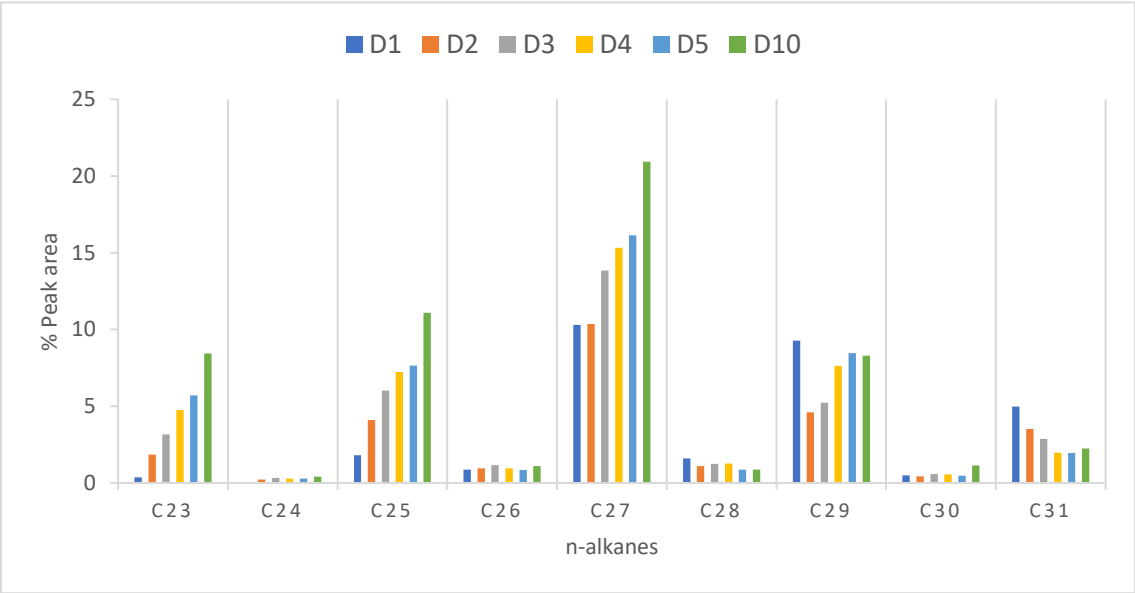
Appendix 23: Plot showing the total concentrations of n-alkanes and branched methyl alkanes present over the empty puparia for *C. vicina* stored indoors



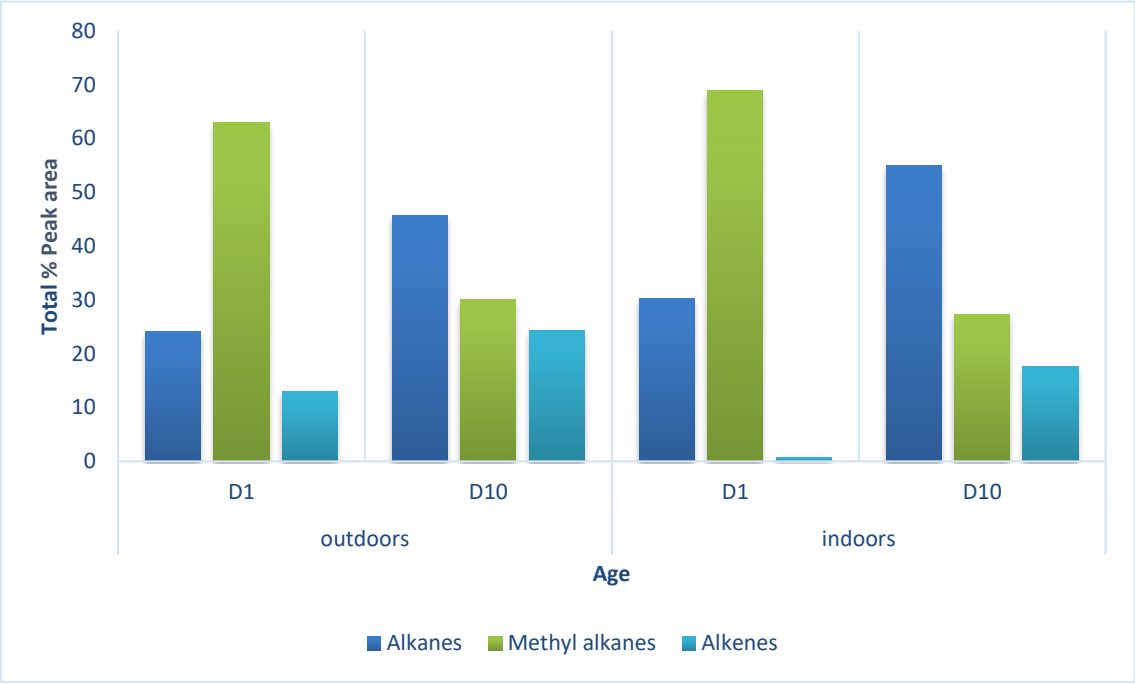
Appendix 24: Plot of the percentage peak area of C23:H to C31:H n-alkanes of adult flies for *L. sericata* reared outdoors



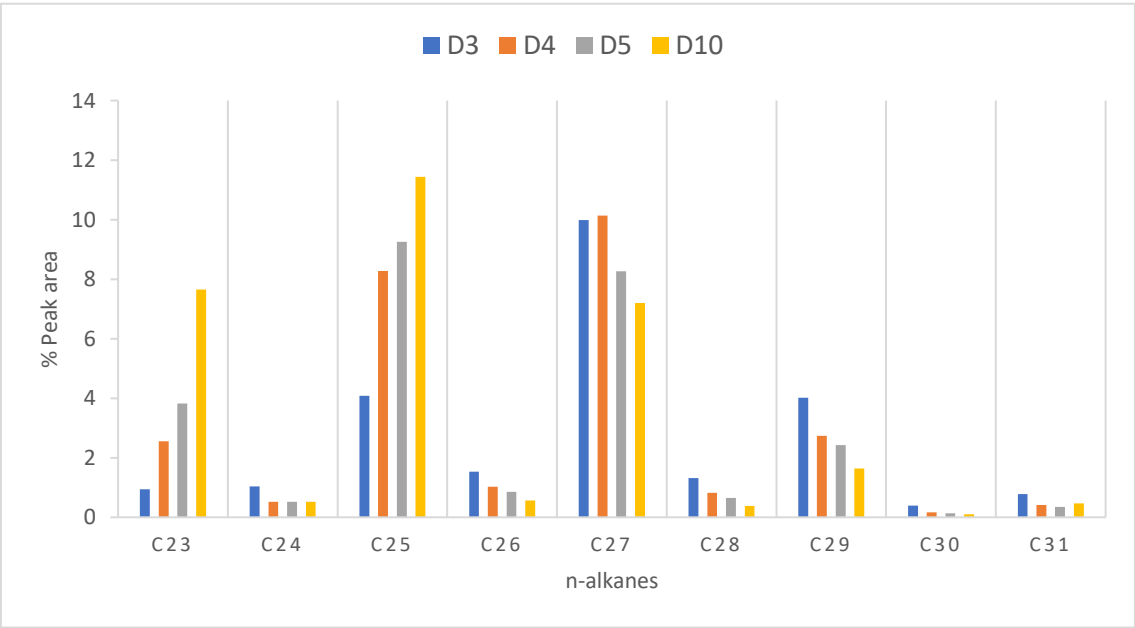
Appendix 25: Percentage peak areas of C23:H to C31:H n-alkanes of *L. sericata* adult flies reared at 25°C, from Day 1 to Day 10



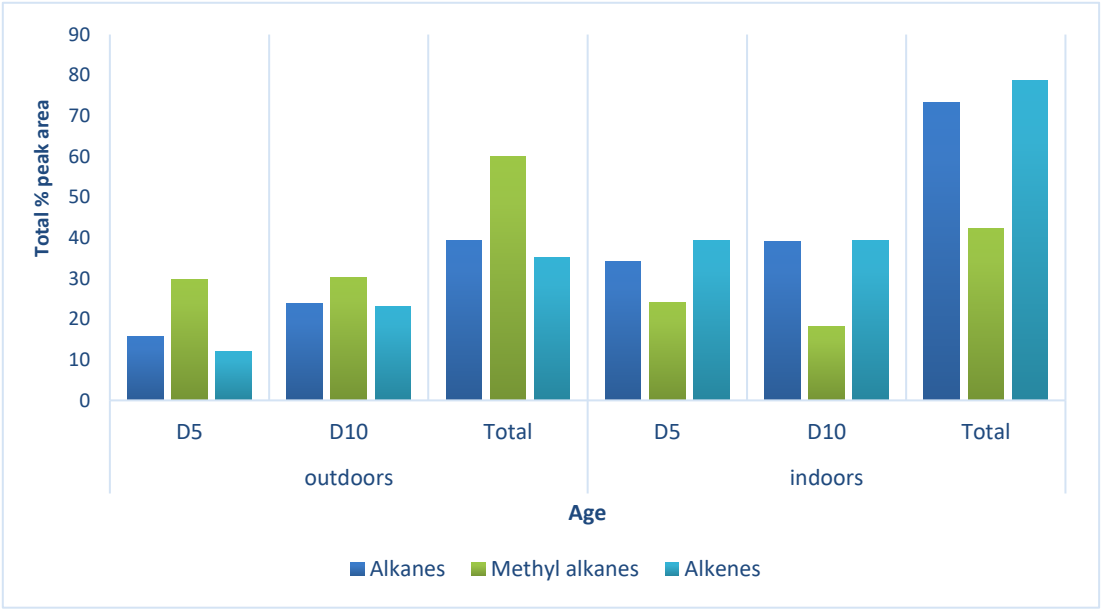
Appendix 26: Plot showing the total percentage concentration of n-alkanes, branched methyl alkanes and alkenes for Day 1 and Day 10 for *L. sericata* in/outdoor adult flies



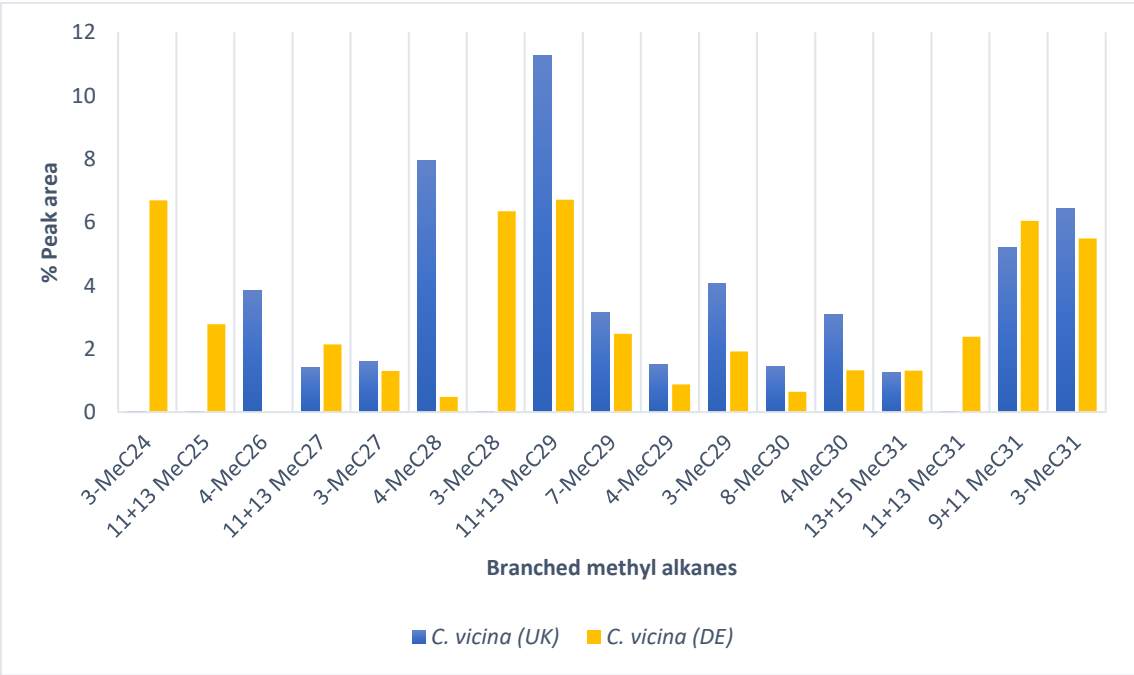
Appendix 27: Percentage peak area of C23:H to C31:H alkanes of *C. vicina* adult flies reared in the UK, for Day 3 to Day 10



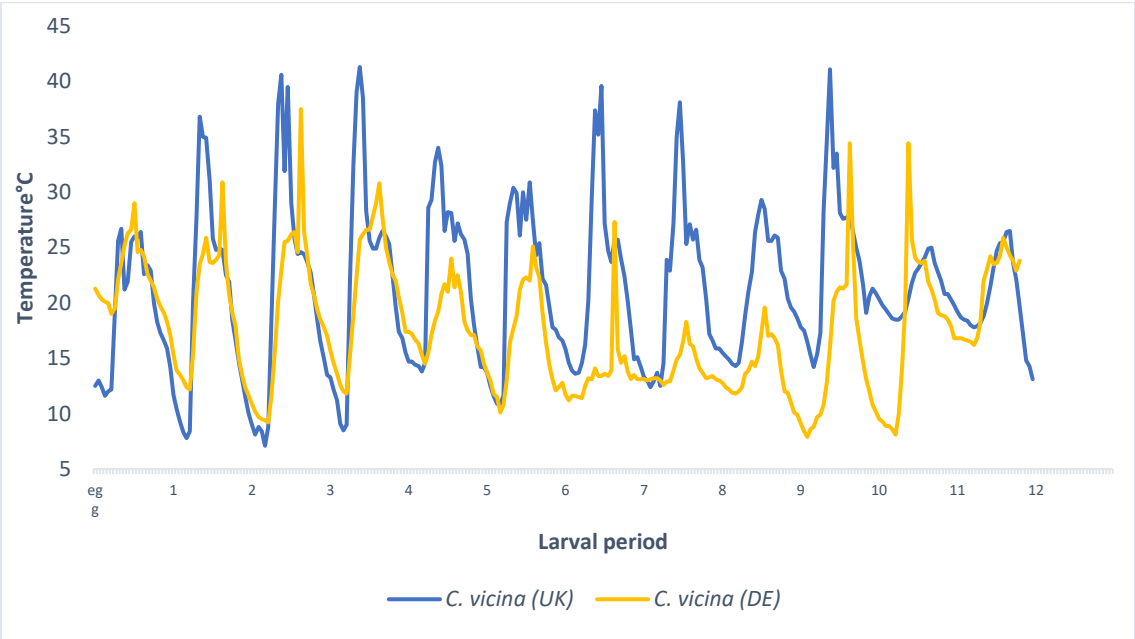
Appendix 28: Plot showing the total concentration of n-alkanes, branched methyl alkanes and alkenes for Day 5 and Day 10 for *C. vicina* in/outdoor adult flies. The total represents the percentage of CHCs from Day 5 and Day 10 extractions for both experimental sets.



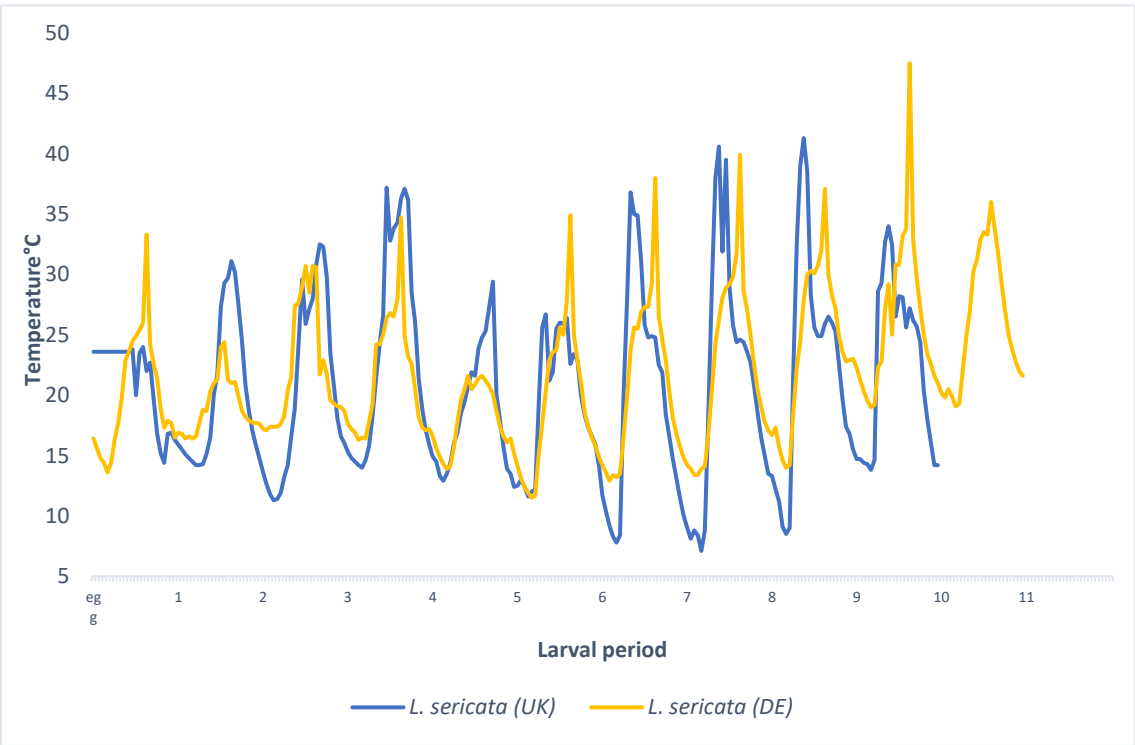
Appendix 29: Percentage concentrations of branched methyl alkanes for *C. vicina* blowfly eggs collected from the UK and Germany



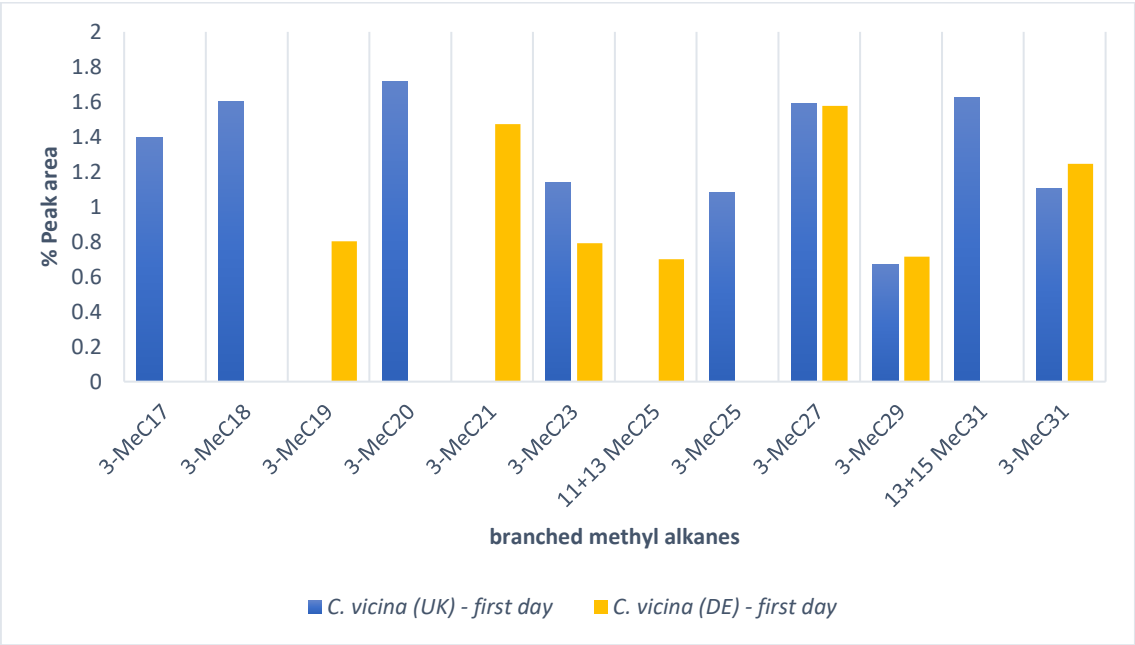
Appendix 30: Comparative weather temperatures in Swindon (UK) and Frankfurt (Germany) during *C. vicina* larval stage



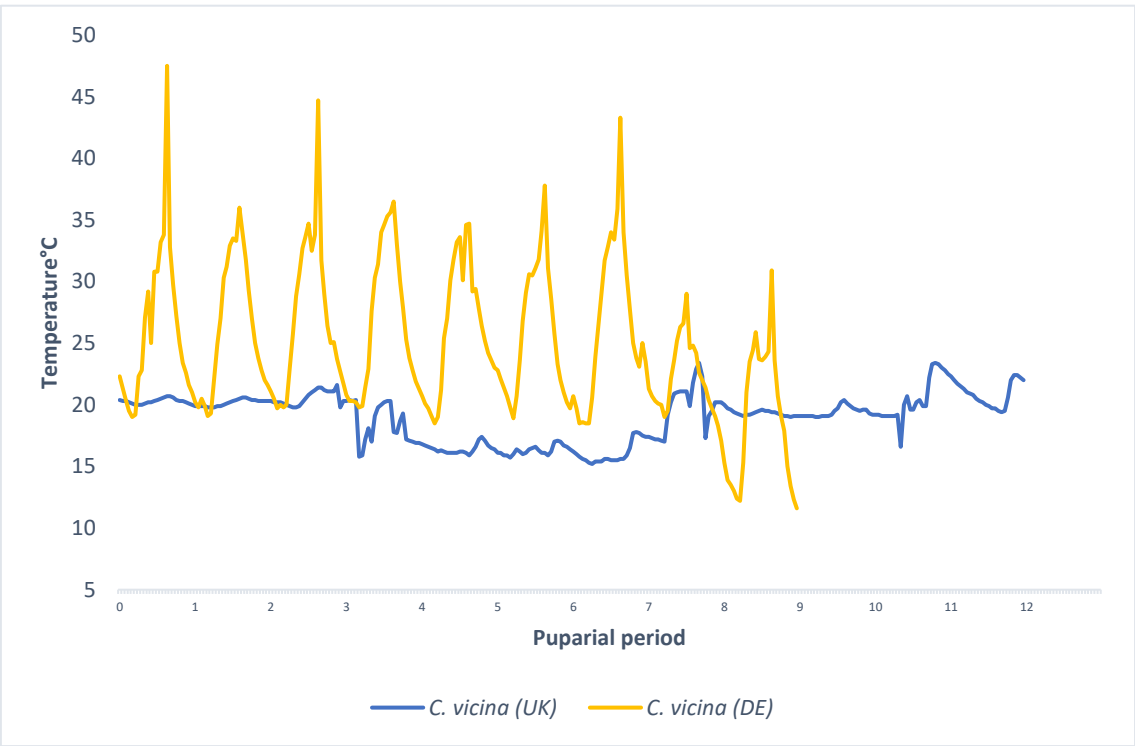
Appendix 31: Comparative weather temperatures in Swindon (UK) and Frankfurt (Germany) during *L. sericata* larval stage



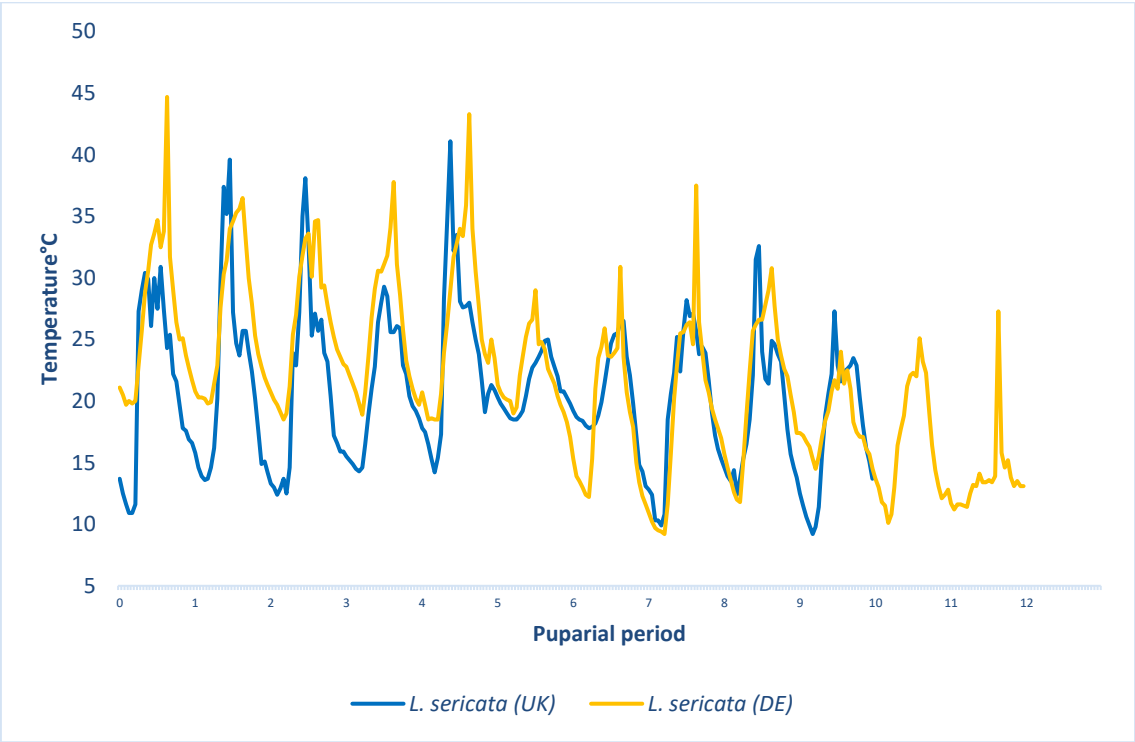
Appendix 32: Plot showing the percentage concentrations of branched methyl alkanes detected in *C. vicina* first day pupae reared outdoors in the UK and Germany



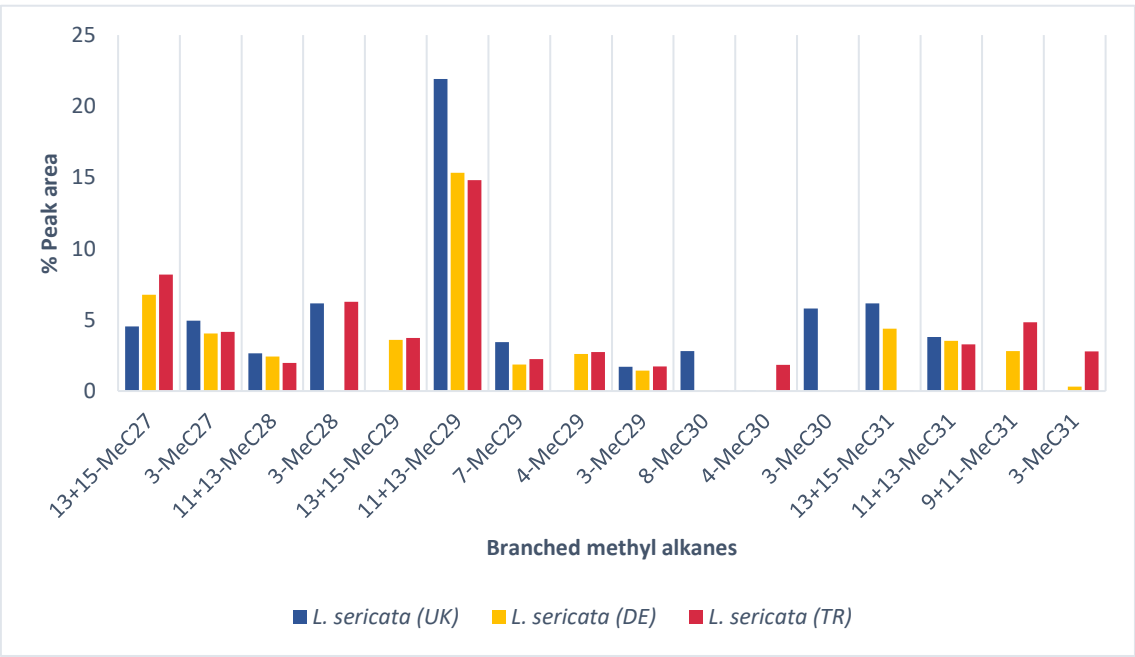
Appendix 33: Comparative weather temperatures in Swindon (UK) and Frankfurt (Germany) during the *C. vicina* pupae stage



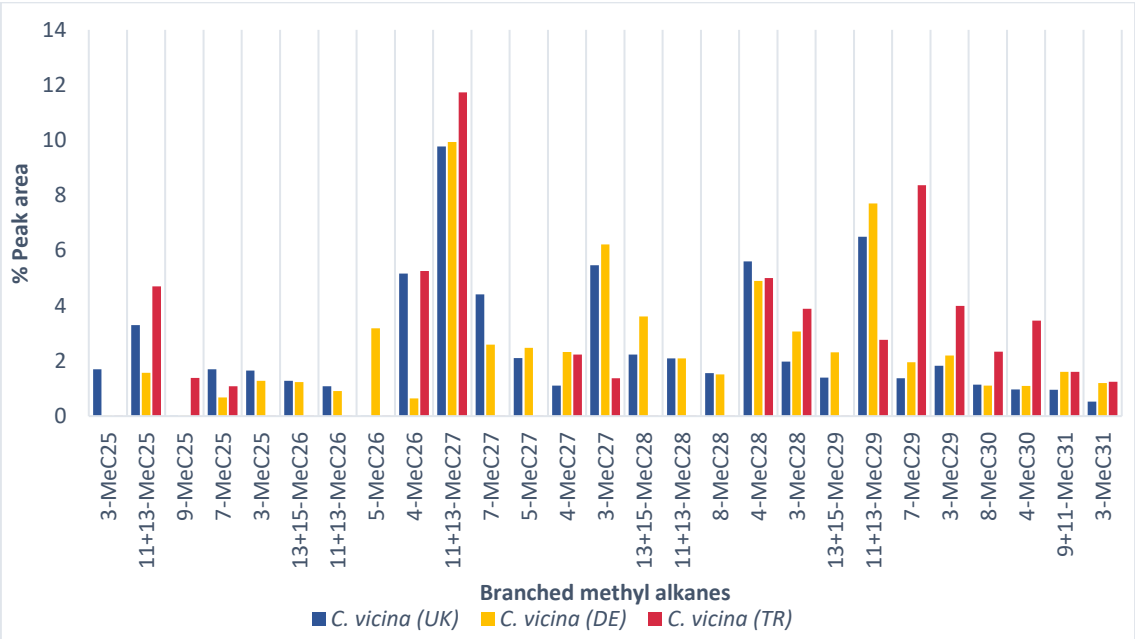
Appendix 34: Comparative weather temperatures in Swindon (UK) and Frankfurt (Germany) during *L. sericata* puparial stage



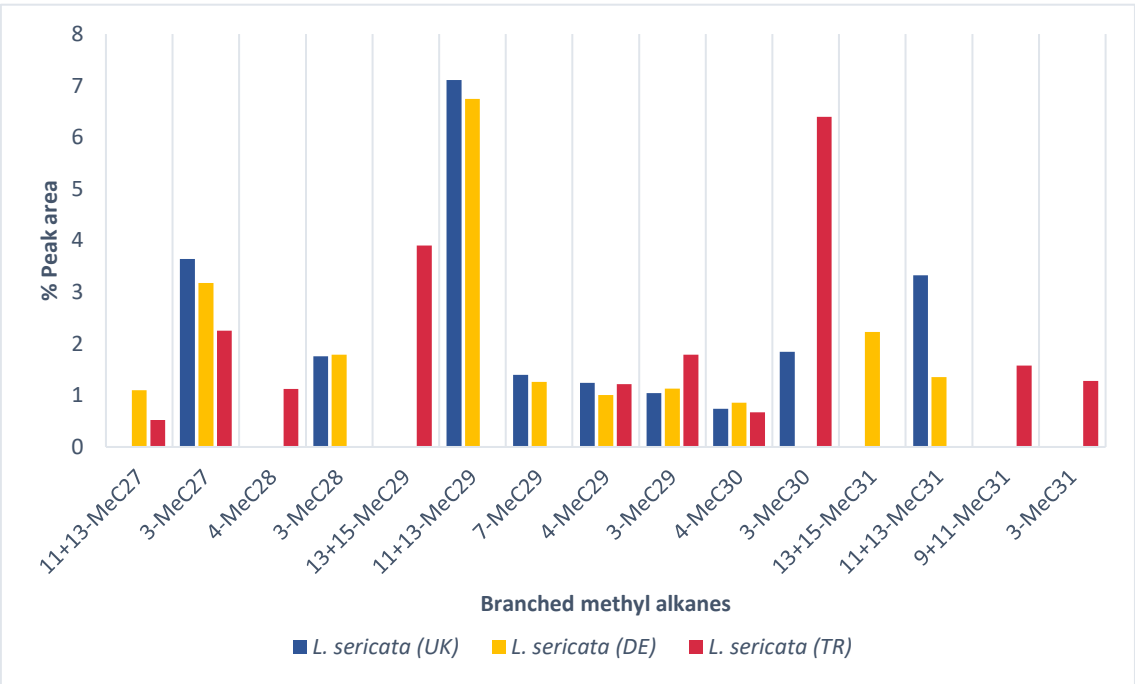
Appendix 35: Plot showing the percentage concentrations of branched methyl alkanes detected in *L. sericata* adult flies collected from the UK, Germany and Turkey



Appendix 36: Plot showing the percentage concentrations of branched methyl alkanes detected in *C. vicina* adult flies collected from the UK, Germany and Turkey



Appendix 37: Plot showing the percentage concentrations of branched methyl alkanes detected in *L. sericata* adult flies collected from the UK, Germany and Turkey



Appendix 38: Plot showing the percentage concentrations of branched methyl alkanes detected in *C. vicina* empty puparia collected from the UK, Germany and Turkey

