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Abstract: Cuticular hydrocarbons were extracted daily from the larvae of two closely related blowflies Calliphora vicina and Calliphora vomitoria (Diptera:Calliphoridae). The hydrocarbons were then analysed using Gas Chromatography-Mass Spectrometry (GC-MS), with the aim of observing changes within their chemical profiles in order to determine the larval age. The hydrocarbons were examined daily for each species from 1 day old larvae until pupariation. The results show significant chemical changes occurring from the younger larvae to the post-feeding larvae. With the aid of a multivariate statistical method (Principal Component Analysis and Artificial Neural Networks), samples were clustered and classified, allowing for the larval age to be established. Results from this study allowed larvae to be aged to the day with at worst, 87% accuracy, which suggests there is great potential for the use of cuticular hydrocarbons present on larvae to give an indication of their age and hence potentially a valuable tool for minimum PMI estimations.

Acknowledgements

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Age estimation of Calliphorida (Diptera: Calliphoridae) larvae using cuticular hydrocarbon analysis and Artificial Neural Networks

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***Highlights (for review)**

- An ageing technique of forensically important larvae using cuticular hydrocarbons
- Analysed using Gas Chromatography – Mass Spectrometry
- Statistically analysed using Principal Component Analysis and Artificial Neural Networks
- Successfully age larvae of *Calliphora vicina* and *Calliphora vomitoria*

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Abstract

Cuticular hydrocarbons were extracted daily from the larvae of two closely related blowflies *Calliphora vicina* and *Calliphora vomitoria* (Diptera:Calliphoridae). The hydrocarbons were then analysed using Gas Chromatography-Mass Spectrometry (GC-MS), with the aim of observing changes within their chemical profiles in order to determine the larval age. The hydrocarbons were examined daily for each species from 1 day old larvae until pupariation. The results show significant chemical changes occurring from the younger larvae to the post-feeding larvae. With the aid of a multivariate statistical method (Principal Component Analysis and Artificial Neural Networks), samples were clustered and classified, allowing for the larval age to be established. Results from this study allowed larvae to be aged to the day with at worst, 87% accuracy, which suggests there is great potential for the use of cuticular hydrocarbons present on larvae to give an indication of their age and hence potentially a valuable tool for minimum PMI estimations.

Keywords: cuticular hydrocarbons, GC-MS, PCA, ANN, Calliphoridae, larvae, minimum PMI estimations.

Introduction

The main contributing factors required to establish the minimal Post-Mortem Interval (PMI_{min})

are species identification and age determination of necrophagous fly larvae [1]. Since Calliphoridae species are known to be the first colonisers of decomposing remains in most circumstances, they are of great forensic importance and have been widely studied [2][3][4]. However, to determine the age of larvae can be challenging and often requires a highly knowledgeable forensic entomologist. The post-feeding larvae also remain to be a problematic stage to age due to the lack of morphological changes occurring with time [5] and therefore the analyst must rely heavily on growth charts. DNA-based analyses have been applied to the field of forensic entomology for over a decade [6]. They have been successfully used to identify and age forensically important species [7-11].

One technique that may have the potential to give the same accuracy as DNA-based techniques for an ageing tool is Cuticular Hydrocarbon (CHC) analysis [12].

CHCs are found in the lipid wax layer of nearly all insects. They have different functions in different species of insects and previous studies have shown their use for age estimation [1, 13-16], sex [17] and species identification [18-20].

The two main factors believed to be influential for the composition of hydrocarbon pools are development/genetic factors and physiological state/environmental conditions [21-23]. The changes observed during the blowflies' development may be affected by the environment they are exposed to. Larvae develop in warm, humid conditions (decomposing remains) and in this stage of their life cycle, they yield profiles consisting of a mixture of low and high molecular weight hydrocarbons.

CHC analysis has the potential to be able to age larvae as they develop through the larval life stages with a good degree of accuracy [1, 13-14, 24]. These characteristics therefore hold a lot of potential in the field of forensic entomology for PMI estimations. CHCs have the advantage of determining the age to the day, rather than to the life stage and compliment the current techniques used in the field. For more information on CHCs, the reader is referred to the following references [25-27].

Artificial neural networks (ANNs) are a machine learning approach that has been utilised

extensively in a variety of applications for pattern recognition and clustering. They have the ability to learn characteristics contained in a dataset and use this to recognise and classify new, previously unseen, data. ANN learning is achieved by altering the weighted connections between each of the artificial neurons contained within the network. This process of weight changes usually occurs until a suitable error (defined as the difference between the actual output of the network and the ground truth target output) is reached. ANNs are well-suited to processing noisy non-linear data and have the ability to learn the relationship between input and output data, making them well-suited to fast processing of large real-world datasets (e.g. speech recognition [28], robotics [29], structural engineering [30] as well as the forensic sciences [31].

Of the many types of ANNs that exist, the Self Organising Map (SOM) [32] is an unsupervised approach that clusters training data based on its underlying characteristics. A SOM consists of an input and an output layer containing artificial neurons, where each neuron in one layer is connected to every neuron in the next. The SOM maps multidimensional data with similar characteristics into topologically co-located clusters in its output layer, generating clusters which represent similar data points in its output layer once training is complete.

Briefly, the unsupervised training of a SOM involves the presentation of every input pattern to the input layer where the incoming weighted connections of the output neuron which best matches the input pattern (known as the winning neuron) are modified. This change in weights results in the formation of clusters within the output layer where similar input patterns cluster in close proximity in the output layer. Readers are referred to [32] and [33] for further details on SOM training.

The topological ordering capabilities of a SOM make it well suited to the analysis of high dimensional data such as that collected from blowfly species. This was shown in a previous study where the classification of hydrocarbon data collected from *Lucilia sericata* pupae was classified using a SOM providing classification accuracy that exceeded 89% [16].

The novelty of this study is to examine the CHC profiles of the larvae from two forensically

important blowfly species found in the UK, *Calliphora vicina* and *Calliphora vomitoria*, with the aim of determining whether there are distinguishable chemical changes occurring over time and to test the reliability of the method by investigating whether this technique is more generally applicable across species. Gas Chromatography-Mass Spectrometry (GC-MS) was used to analyse the HCs and methods of statistical and artificial intelligence data analysis were applied to the compiled datasets to cluster and automatically classify the data as well as aid data visualisation.

Material and Materials

Insect materials

A colony of *Calliphora vicina* and *Calliphora vomitoria,* (geographical origin, University of Birmingham campus, UK), kindly supplied by the Scott Hayward's research group at the University of Birmingham, was reared in the laboratory and maintained in separate rearing cages under standard environmental conditions (22□1 ^{o}C). They were fed with sugar and water on a need-to basis and pigs liver, which was used as an oviposition medium. Once the eggs were laid they were separated into plastic containers containing approximately 700 eggs. This ensured there would be enough larvae for the completion of the life cycle with daily extractions and to resolve the problem of overcrowding and food competition. The larvae were fed daily with minced beef (approximately 50 g) and were kept in an incubator at a set temperature of 22±1 °C. Under the rearing temperature of 22±1 °C larvae reached the puparial stage after 11 days for C. vicina and 14 days for C. vomitoria. The hydrocarbons were extracted daily from the larvae upon hatching, until they pupariated.

Sample Preparation

Liquid extraction with hexane was used to extract the hydrocarbons. For each sample (n=10) a number of larvae (Table 1) were pooled together to ensure the concentration was sufficient for the GC-MS to detect the hydrocarbons [12]. Preliminary experiments carried out at the rearing

temperature showed that approximately 20-30 first instar larvae yielded a sufficient concentration to produce a reliable chromatogram on the GC-MS. As the larvae became older, and therefore larger in size, fewer larvae were used for the extractions (Table 1). The larval sample numbers were reduced as they aged due to the hydrocarbon concentration increasing with larval size and therefore preventing the GC-MS from overloading. The larvae were placed into a GC vial with hexane ensuring that they were submerged. It was left for 10 to 15 minutes after which the hexane was removed and passed through a silica gel column. The column was made by plugging a Pasteur pipette with glass wool followed by a small amount of silica gel. The larval extract from the GC vial was transferred to the column and an additional 500 µl of hexane was added. The eluted hexane was collected into a clean GC vial and left until completely dry. The extracts were redissolved in 10 µl (for manual injections) and 20 µl (for autosampler injections) of hexane and a 2 µl aliquot was injected into the GC-MS. For the first 3 days extracts were injected manually and thereafter with the autosampler.

Table 1: Number of larvae used daily during *C. vicina* and *C. vomitoria* larval extractions

Species	Day	Instar	Number of larvae added to each sample *n=10*
C. vicina	1	1st instar	~20
	2	2nd instar	10
	3	3rd instar	5
	4 & 5	3rd instar	3
	6 to 11	3rd instar & post- feeding	2
C. vomitoria	1	1st instar	~30
	2	2nd instar	~15
	3	2nd/3rd instar	7
	4 & 5	3rd instar	3
	6 & 10	3rd instar	2
	11	Post-feeding	2
	12 to 14	Post-feeding	1

Chemical analysis of extracts

Chemical analysis of all extracts was carried out on an Agilent Technologies 6890N Network GC with a split/splitless injector at 250 ^{o}C, a Restek Rxi-1MS capillary column (30m x 0.25 mm ID, 0.25µm film thickness) and coupled to an Agilent 5973 Network Mass Selective Detector. The GC was coupled to a computer and data processed with Agilent Chemstation software. Elution was carried out with helium at 1mL/min. The oven temperature was programmed to be held at 50 ^{o}C for 2 minutes then ramped to 200 ^{o}C at 25 ^{o}C/min, then from 200 ^{o}C to 260 ^{o}C at 3^{o}C/min and finally from 260 ^{o}C to 320 ^{o}C at 20 ^{o}C/min where it was held for 2 minutes. The mass spectrometer was operated in Electron Ionisation mode at 70 eV, scanning from 40 – 500 amu at 1.5 scans s^{-1}. Hydrocarbons were identified using a library search (NIST08), the diagnostic fragmented ions and the Kovats indices.

Statistical analysis:

Principal Component Analysis

The chromatograms were initially interpreted using Principal Component Analysis (PCA) in order to ease visualization of trends that may be present within the dataset. For further details the reader is referred to [12, 34]. PCA has already been used by the authors to age *Lucilia sericata* [13] and the same experimental design was used for this study. Methyl branched alkanes and alkenes with a percentage peak area greater than 0.5% were used for statistical analysis.

Artificial Neural Networks

The same training and testing approach reported in [16] is used in the current study to automatically classify the larvae of both *Calliphora vicina* and *Calliphora vomitoria*, where readers are referred to for further details. Briefly, the data was preprocessed using PCA to reduce the dimensionality of the data, with six principal components that contained the most variance used (percentage sum of Eigenvalues for PC1 to PC 6 - *C. vicina* 96% and *C. vomitoria* 97%) as input data for the neural network. The data was then normalized between the range -1 and +1 by:

$$v_n = \frac{v - \min(v)}{\max(v) - \min(v)} \times (U_{lim} - L_{lim}) - L_{lim}$$

where v_n is the normalised data, v is the original data, $max(v)$ and $min(v)$ are the maximum and minimum data values respectively and $_{Ulim}$ and $_{Llim}$ are the desired upper and lower limits of the normalised data which in this study are set to +1 and -1 respectively. Training data was presented to the SOM by averaging five hydrocarbon profiles for both spec ies' larvae and adult flies. Testing the generalization performance of the SOM was estimated using two approaches:

1) Presenting the remaining unseen individual hydrocarbon profiles to the SOM
2) Presenting the average of the remaining unseen hydrocarbon profiles to the SOM

Training was performed using 10-fold cross-validation and an average and standard deviation across the ten folds calculated. For each fold, a random subset for each day's

profile was chosen for the training and testing data, with a different subset chosen for each fold (note: the same subsets were chosen for both testing approaches). In this study, two important SOM training parameters, the neighbourhood size and learning rate, were updated during training after a set number of elapsed epochs as described in Day et al [33]. A number of candidate output layer sizes were systematically evaluated to find the output layer size that delivered the most effective clusters.

Results

GC-MS analysis:

C. vicina

C. vicina yielded a profile of 46 different identifiable compounds with some co-eluting resulting in a total of 40 resolvable peaks from day 1 to 11 (Table 2). All compounds were hydrocarbons consisting of *n*-alkanes (33%), alkenes (17%), and methyl branched hydrocarbons (50%) for day 1. The chain lengths ranged from C20:H to C33:H.

The lower molecular weight compounds in the profile (C20:H to C22:H) are mainly made up of volatile compounds which are chemically less stable and variable, therefore they were not used for subsequent PCA and ANN analysis as they showed little significance in ageing the larvae. The middle region of the chromatogram consists of straight chain *n*-alkanes, alkenes and methyl branched alkanes (ranging from C23:H to C27:H). The higher end of the chromatogram is dominated by high boiling point *n*-alkanes (ranging from C29:H to C33:H) and methyl branched alkanes which are at their most abundant in the 1st and 2$_{nd}$ instar larvae.

Table 2 List of all compounds extracted from the larvae of *C. vicina* and their calculated Kovats Indices to aid identification. Compounds in bold were used for subsequent PCA analysis (peak numbers refer to numbers in Figure 1)

Peak number	Peak Identification	Kovats iu
1	**Eicosene**[1]	1990
2	Eicosane	2000
3	Heneicosane	2100
4	**Docosene**[1]	2190
5	Docosane	2200
6	Tricosane	2300
7	**7-Methyltricosane**	2342
8	**5-Methyltricosane**	2351
9	**3-Methyltricosane**	2373
10	Tetracosane	2400
11	**2-Methyltetracosane**	2465
12	**Pentacosene**[1]	2471
13	**Pentacosene**[1]	2479
14	Pentacosane + Phthalate	2500
15	**11-Methylpentacosane**	2536
16	**9-Methylpentacosane**	2538
17	**7-Methylpentacosane**	2544
18	**5-Methylpentacosane**	2552
19	**3-Methylpentacosane**	2574
20	Hexacosane	2600
21	**x,12-Dimethylhexacosane**[2] **+ Heptacosene**[1]	2666
22	**Heptacosene**[1]	2676
23	**Heptacosene**[1]	2679
24	Heptacosane	2700
25	**11+13-Methylheptacosane**	2735
26	**7-Methylheptacosane**	2743
27	**5-Methylheptacosane**	2753
28	**3-Methylheptacosane**	2775
29	Octacosane	2800
30	**2-Methyloctacosane**	2871
31	Nonacosane	2900
32	**11+13-Methylnonacosane**	2936
33	**9-Methylnonacosane**	2941
34	**7-Methylnonacosane**	2947
35	**3-Methylnonacosane**	2977
36	Triacontane	3000
37	**2-Methyltricontane**	3067
38	**2,6/2,8/2,10-Dimethyltriacontane**[2]	3097

39	Hentriacontane	3100
40	Tritriacontane	3200

[1]Double bond position assumed but not assigned to specific peaks

[2]Tentative Identification based on calculated Kovats Indicies values and match with NIST08 Library database

Figure 1 shows the stacked GC chromatograms of a single sample (Table 1) from larvae extracted at days 1, 5, 7 and 11. The shaded bars highlight areas of contrast within the profiles showing potential for ageing the larvae of *C. vicina* because of the distinguishable chemical changes occurring with time.

The profile of the 1st instar (day 1) possesses no peaks specific to this stage. The 2nd instar, represented in day 2, also reveals no age specific compounds but there are eight peaks specific to both the 1st and 2nd instar stage (peak 26, 27, 32-34, 37, 38). The number of methyl branched compounds is greatly reduced from the immature larvae stages (1st and 2nd) to the 3rd instar. This can be seen in Figure 2 in the highlighted areas on the two chromatograms of A (day 1-1st instar) and B (day 5 – 3rd instar).

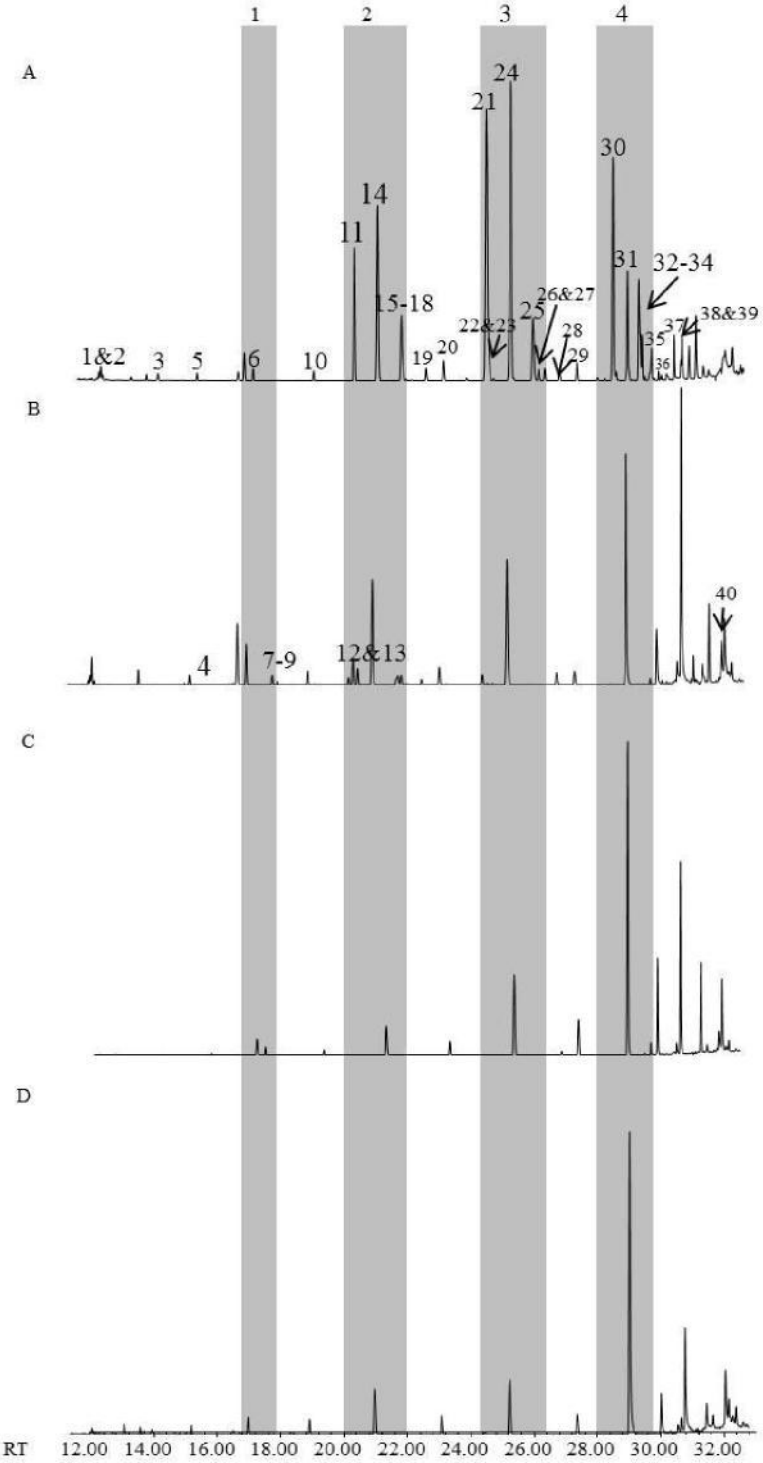

Figure 1: GC chromatograms of *C. vicina* larvae at four different ages, A: Day 1, B: Day 5, C: Day 7 and D: Day 11. Shaded bars illustrate distinctive changes over time indicating specific areas of interest

Day 3 to day 5 reveal very similar chromatograms but they can be aged to the day rather than to the instar (3rd instar) because of the varying peak area ratios (Table 4). This instar has three compounds that are specific to this phase, 7-MeC23:H, 5-MeC23:H and 3-MeC23:H. This group of MeC23:H isomers (peak 7 to 9 in Figure 1) could be a very good age indicator for the 3rd instar stage, with the 7-MeC23:H (peak 7) also increasing further with age during the 3rd instar.

Peak 21 (x,12-DimeC26:H) co-elutes with C27:1 in the younger larvae (up to day 2), making it a good age indicator. C21:1 and C22:1 (peaks 1 & 4) both increase gradually as the larvae age (up to 23% and 16% respectively), before decreasing in the late post-feeding stage at day 11.

GC-MS analysis: C. vomitoria

C. vomitoria exhibited a profile of 57 identifiable compounds with some co-eluting giving a total of 51 resolvable peaks (Table 5). Of these 51 resolvable peaks, 96% were hydrocarbons, with day 1 consisting of *n*-alkanes (28%), alkenes (29%) and mono-methyl alkanes (43%). The chain length of all hydrocarbons range from C16:H to C33:H.

Table 3: List of all compounds from C20:H extracted from the larvae of *C. vomitoria* and their Kovats Indices to aid identification. Compounds in bold were used for subsequent PCA (peak numbers refer to numbers in Figure 3)

Peak Number*	Peak Identification	Kovats iu
1	Eicosane	2000
2	Heneicosene[1]	2066
3	Heneicosene[1]	2074
4	Heneicosane	2100
5	**3-Methylheneicosane**	2172
6	Docosane	2200
7	**2-Methyldocosane**	2264
8	Tricosene[1]	2271
9	Tricosene[1]	2278
10	Tricosane	2300
11	**9+11-Methyltricosane**	2339
12	**7-Methyltricosane**	2343
13	**5-Methyltricosane**	2348
14	**3-Methyltricosane**	2374
15	Tetracosane	2400
16	**2-Methyltetracosane**	2464
17	Pentacosene[1]	2471
18	Pentacosene[1]	2478
19	Pentacosane	2500
20	**9+11-Methylpentacosane**	2536
21	**7-Methylpentacosane**	2539
22	**5-Methylpentacosane**	2549
23	**3-Methylpentacosane**	2574
24	Hexacosane	2600
25	**2-Methylhexacosane**	2665
26	Heptacosene[1]	2669
27	Heptacosene[1]	2679
28	Heptacosane	2700
29	**9+11-Methylheptacosane**	2735
30	**3-Methylheptacosane**	2774
31	Octacosane	2800
32	**2-Methyloctacosane**	2871
33	Nonacosene[1]	2879
34	Nonacosene[1]	2886
35	Nonacosane	2900
36	**11+13-Methylnonacosane**	2937
37	**7-Methylnonacosane**	2948

38	**5-Methylnonacosane**	2957
39	**Dimethylnonacosane**	2966
40	**3-Methylnonacosane**	2978
41	Tricontane	3000
42	Cholesterol + Hentriacontene[1]	3070
43	Hentriacontene[1]	3077
44	Hentriacontene[1]	3085
45	Hentriacontane	3100
46	**11+15-Methylhentriacontane**	3131
47	**3-Methylhentriacontane**	3175
48	Dotriacontane	3200
49	Tritriacontene[1]	3263
50	Tritriacontene[1]	3276
51	Tritriacontane	3300

[1] Double bond position assumed but not assigned to specific peaks

Figure 2 shows the chromatograms of a single sample (Table 1) from days 1, 4, 8 and 13. Chemical distinctions can be made between the different ages from the chromatogram comparison over time. The shaded bars highlight some regions of contrast within the profiles of the different ages.

Again, the lower retention time compounds of the profile are mainly made up of volatile compounds which are less stable and therefore excluded from PCA and ANN analysis. As with the chemical profile of *C. vicina*, the middle section of the chromatogram consists of *n*alkanes, alkenes and methyl branched alkanes (ranging from C23:H to C27:H). The higher end of the chromatogram is dominated by high molecular weight *n*-alkanes (ranging from C29:H to C33:H) and methyl branched alkanes which are at their most abundant in the 3rd instar larvae.

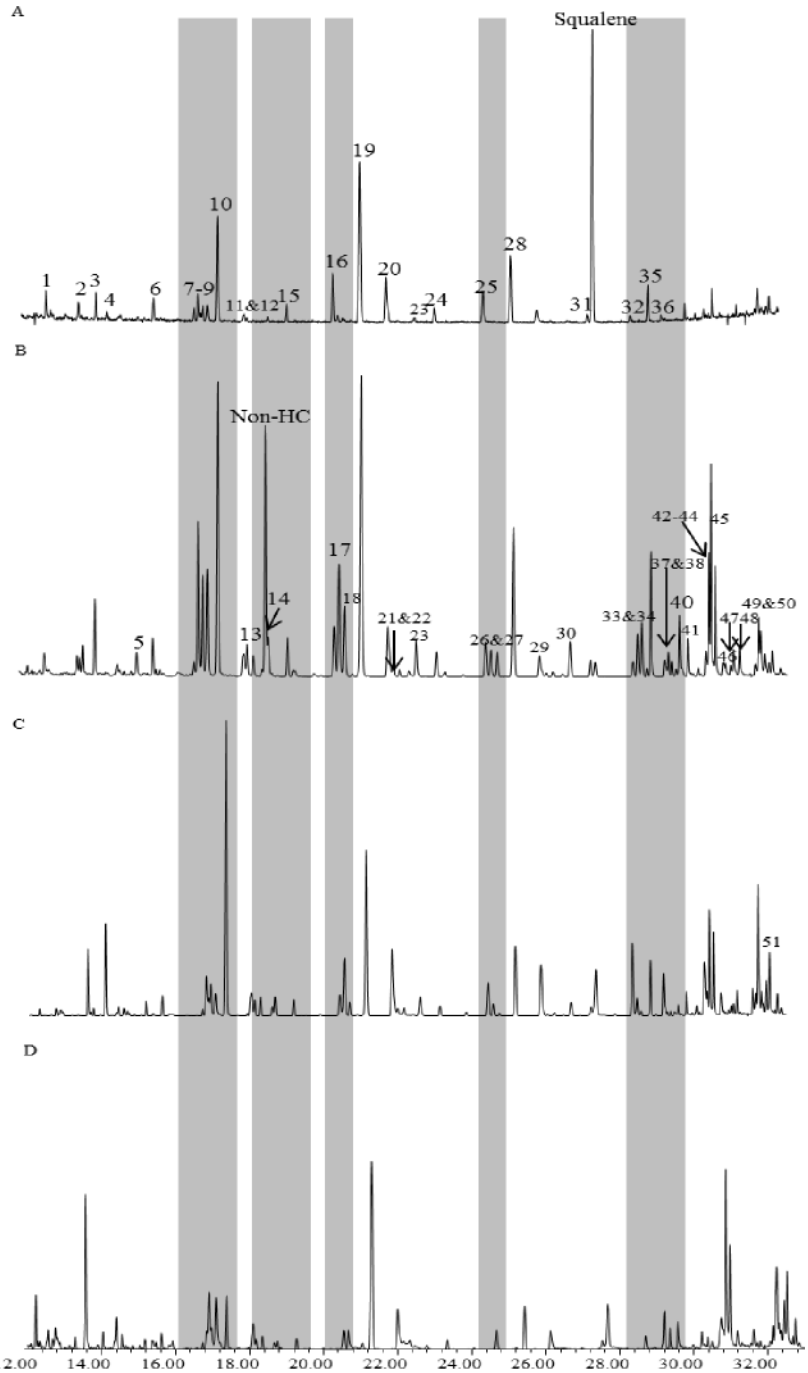

Figure 2. GC chromatograms of *C. vomitoria* larvae at four different ages, A: Day 1, B: Day 4, C: Day 8 and D: Day 13. Shaded bars illustrate distinctive changes over time indicating specific areas of interest

There are no compounds exclusive to the profile of day 1 larvae (1st instar). However, there are a few compounds specific to certain larval ages, such as 3-MeC29:H and 3-MeC31:H, which are only seen with a relative peak area percentage greater than 0.5% in day 4. 3-MeC27:H and an unidentified DimeC29:H are specific to day 9 larvae (in a peak area exceeding 0.5%). These compounds are therefore likely to be good age indicators for these two larval ages.

2-MeC22:H is only present in the early larval life stages (days 1 to 3) and decreases with age so the presence of this compound could be used to determine the early larval life age (1st and 2nd instar). 7-MeC23:H and 3-MeC23:H (Table 4) are present in a high concentration in day 3. 2-MeC24:H is present in substantial concentrations in days 1 and 2 then decrease with age, before an increase is seen in the late post-feeding stage. 9+11-MeC25:H and 2-MeC26:H also appear in a very high concentration in the immature larvae stages (days 1 and 2). Three peaks are absent (below the peak area threshold of 0.5%) from the 1st and 2nd instar larvae (3-MeC21:H, 9+11-MeC27:H and 11+15-MeC31:H) and three compounds are absent from the 1st instar larvae alone (compounds 5-MeC23:H, 3-MeC23:H and 7-MeC25:H). The absence of these compounds from the immature life stages makes them good age indicators.

A group of MeC23:H compounds (9+11-MeC23:H, 5-MeC23:H and 3-MeC23:H) increases significantly in the post-feeding stage (days 10 to 14), implying that they could act as a good post-feeding stage indicator. 7-MeC23:H is not present in a detectable concentration in the late post-feeding stage (days 10 and 11), which again could potentially be a useful age indicator for this life stage. 11+15-MeC31:H is relatively stable over the first three instars after which a considerable increase in the peak area is observed in the post-feeding stage.

Principal Component Analysis:

C. vicina:

PCA analysis was initially carried out including all three classes of hydrocarbons (*n*-alkanes, alkenes and methyl branched alkanes). However, this gave a lot of scatter and cluster overlap such that an accurate age could not be determined for individuals. A dataset containing just the alkenes and methyl branched alkanes was therefore compiled.

Of the 40 resolvable peaks extracted from the cuticle of *C. vicina* larvae, 27 hydrocarbon peaks were used for PCA analysis, of which 76% were methyl branched and 24% were alkenes. Table 4 shows the compounds used for PCA analysis, along with the total percentage of each compound present, the percentage standard deviation for each day and the calculated Kovats Indices.

Table 4: List of the compounds extracted and used for subsequent PCA analysis from the larvae of *C. vicina*, with the total percentage of each compound present, the percentage standard deviation for each day and the calculated Kovats Indices to aid identification.

Peak number	Peak Identification	Kovats iu	Day 1 *n*=10 %	Day 2 *n*=10 %	Day 3 *n*=10 %	Day 4 *n*=10 %	Day 5 *n*=10 %	Day 6 *n*=10 %	Day 7 *n*=10 %	Day 8 *n*=10 %	Day 9 *n*=10 %	Day 10 *n*=10 %	Day 11 *n*=10 %
1	Eicosene[1]	1990	1.79±0.68	3.52±1.54	5.48±2.47	3.55±0.64	4.55±2.12	7.19±3.07	6.61±1.36	9.62±2.60	23.23±17.18	23.77±5.38	14.59±12.54
4	Docosene[1]	2190	tr	tr	tr	tr	3.26±1.51	5.52±1.35	5.79±1.24	8.59±2.47	17.03±10.91	16.43±4.43	11.93±9.49
7	7-Methyltricosane	2342	tr	tr	4.43±2.13	4.76±1.49	5.60±3.23	tr	tr	tr	tr	tr	tr
8	5-Methyltricosane	2351	tr	tr	2.44±1.23	2.21±0.76	2.92±1.77	tr	tr	tr	tr	tr	tr
9	3-Methyltricosane	2373	tr	tr	2.48±1.25	tr	2.79±1.55	tr	tr	tr	tr	tr	tr
11	2-Methyltetracosane	2465	12.55±4.83	8.12±5.46	5.07±3.02	4.40±1.40	5.05±3.13	4.75±1.16	8.14±2.93	13.23±9.07	20.76±13.98	15.78±5.01	18.20±15.51
12	Pentacosene[1]	2471	tr	tr	13.88±7.34	14.89±5.04	12.35±6.91	13.06±5.15	tr	tr	tr	tr	tr
13	Pentacosene[1]	2479	tr	tr	11.24±6.25	11.79±4.36	8.94±5.27	8.99±4.20	tr	tr	tr	tr	tr
15	11-Methylpentacosane	2536	9.90±3.93	5.34±1.26	4.59±2.93	4.17±1.37	4.53±2.49	4.63±1.63	6.78±1.62	8.87±2.63	tr	tr	tr
16	9-Methylpentacosane	2538	2.49±1.08	2.13±0.44	3.74±2.15	4.42±1.69	5.62±3.04	5.33±2.03	4.88±0.78	5.78±1.78	tr	tr	tr
17	7-Methylpentacosane	2544	1.48±0.76	2.66±0.46	4.80±3.12	6.15±2.70	8.43±4.54	7.25±2.50	6.21±1.73	tr	tr	tr	tr
18	5-Methylpentacosane	2552	1.16±0.60	1.63±0.68	2.09±1.47	2.49±1.55	3.80±2.98	4.42±1.98	3.38±1.73	tr	tr	tr	tr
19	3-Methylpentacosane	2574	2.19±1.21	2.41±0.87	4.06±2.68	4.97±1.97	4.92±2.66	4.90±1.78	5.24±1.21	5.76±1.95	tr	tr	tr
21	x,12-Dimethylhexacosane[2] + Heptacosene	2666	37.91±26.98	38.39±25.62	17.51±11.18	14.61±4.73	8.87±4.84	9.41±2.44	16.12±5.84	29.33±15.37	38.97±22.46	44.02±15.38	55.28±39.23
22	Heptacosene[1]	2676	1.97±0.88	tr	tr	4.79±1.70	4.30±2.50	tr	tr	tr	tr	tr	tr
23	Heptacosene[1]	2679	0.58±0.35	1.95±0.31	4.32±2.31	4.56±1.96	3.12±1.73	tr	tr	tr	tr	tr	tr
25	11+13-Methylheptacosane	2735	8.29±3.64	6.45±4.78	3.98±2.00	tr	tr	tr	tr	tr	tr	tr	tr
26	7-Methylheptacosane	2743	1.47±0.86	1.67±1.59	tr	tr	tr	tr	tr	tr	tr	tr	tr
27	5-Methylheptacosane	2753	1.37±0.84	1.63±1.52	tr	tr	tr	tr	tr	tr	tr	tr	tr

28	3-Methylheptacosane	2775	1.03±0.86	2.08±1.44	6.15±3.65	8.10±3.06	8.04±4.85	11.47±4.61	17.47±1.83	8.76±3.51	tr	tr	tr
30	2-Methyloctacosane	2871	8.53±7.04	11.28±16.23	3.74±2.65	4.14±2.32	2.91±1.67	4.46±1.21	4.95±1.35	tr	tr	tr	tr
32	11+13-Methylnonacosane	2936	2.75±2.87	3.78±5.39	tr	tr	tr	tr	tr	tr	tr	tr	tr
33	9-Methylnonacosane	2941	1.01±0.97	1.48±1.97	tr	tr	tr	tr	tr	tr	tr	tr	tr
34	7-Methylnonacosane	2947	1.25±1.22	1.85±2.58	tr	tr	tr	tr	tr	tr	tr	tr	tr
35	3-Methylnonacosane	2977	tr	tr	tr	tr	tr	8.63±4.57	14.44±3.31	10.05±7.41	tr	tr	tr
37	2-Methyltricontane	3067	1.46±1.15	2.77±2.34	tr	tr	tr	tr	tr	tr	tr	tr	tr
38	2,6/2,8/2,10-Dimethyltriacontane[2]	3097	0.81±0.92	0.88±0.63	tr	tr	tr	tr	tr	tr	tr	tr	tr

[1]Double bond position assumed but not assigned to specific peaks

[2]Tentative identification based on calculated Kovats Indices values and match with NIST08 Library database

tr = Trace amounts detected <0.5%

PCA was carried out using the first 6 principal components which described 96.9% of the variation within the data set with the first three principal components, comprising 60.5%, 20.3% and 8.9% respectively.

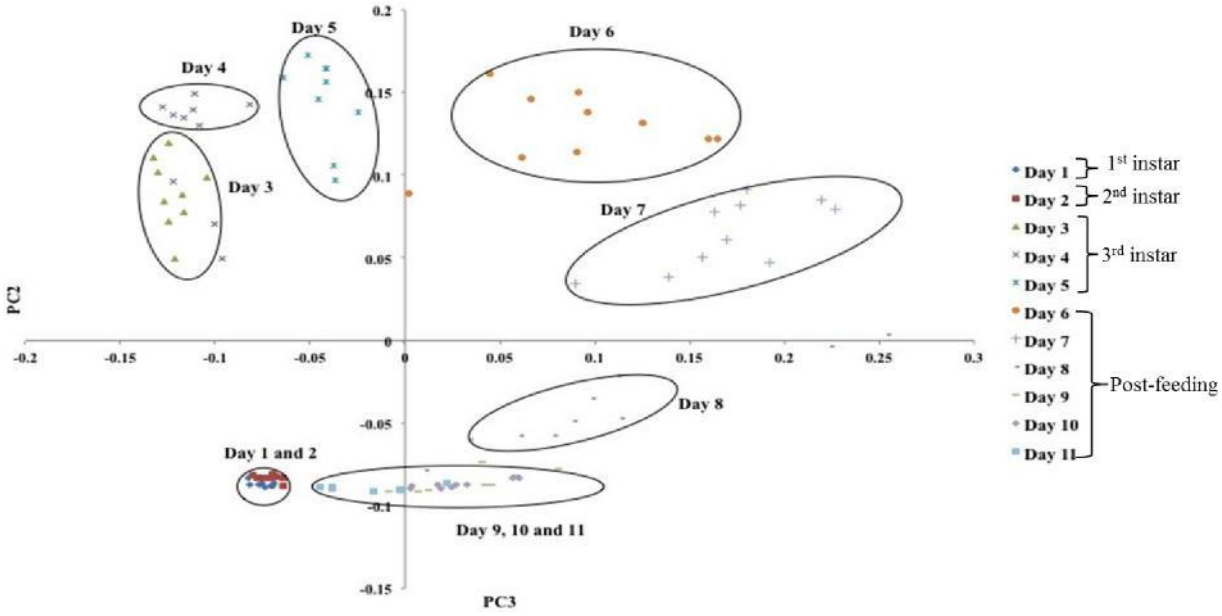

Figure 3: PCA plot showing PC3 against PC2 for *C. vicina* larvae using alkenes and methyl branched alkanes only, with clustering days circled

Figure 3 shows the PCA plot of PC3 vs PC2 for data gathered from day 1 to day 11 of larvae extractions of *C. vicina*. There are eight clusters within the plot allowing for the larvae to be aged down to the day with the exception of days 1 and 2 and the late post-feeding stage (days 9 to 11). The clustering groups follow a systematic pattern clockwise around the plot. Although day 1 has a single compound specific to that day, day 1 and day 2 have eight compounds detectable only in those two life stages (1st and 2nd instar), hence the likely reasoning for these two stages clustering together in the PCA plot. There is a substantial change within the PCA plot from day 2 to day 3, which represents the transition between the 2nd and 3rd instar.

The main compounds which have substantial PCA score values are x,12-diMethylhexacosane which co-elutes with heptacosene in the early larval stages. Another compound exhibiting a high score is 2-Methyltetracosane. Both these methyl branched hydrocarbons are present throughout the larval development of *C. vicina* and this indicates that the methyl branched alkanes are influential for ageing this species.

C. vomitoria:

Of the 51 compounds extracted from the cuticle of *C. vomitoria*, 29 of them were used for subsequent PCA analysis. Table 5 shows the compounds used for PCA analysis, along with the total percentage of each compound present, the percentage standard deviation for each day and the calculated Kovats Indices.

Table 5: List of the compounds extracted and used for subsequent PCA analysis from the larvae of *C. vomitoria*, along with the total percentage of each compound present, the percentage standard deviation for each day and the calculated Kovats Indices to aid identification

Peak no.	Peak Identification	Kovats iu	Day 1 *n*=10 %	Day 2 *n*=10 %	Day 3 *n*=10 %	Day 4 *n*=10 %	Day 5 *n*=10 %	Day 6 *n*=10 %	Day 7 *n*=10 %
5	3-Methylheneicosane	2172	tr	tr	3.97±0.68	3.16±0.61	5.47±2.49	4.27±0.51	3.70±1.00
7	2-Methyldocosane	2264	5.44±2.86	5.06±1.56	2.58±1.05	tr	tr	tr	tr
11	9+11-Methyltricosane	2339	5.32±4.90	6.31±2.67	7.47±2.17	4.96±1.65	8.30±3.96	7.93±1.13	7.47±2.21
12	7-Methyltricosane	2343	2.53±1.13	4.83±1.72	7.31±1.51	4.78±0.89	5.15±2.20	4.33±0.48	3.66±0.84
13	5-Methyltricosane	2348	tr	2.11±0.72	4.53±0.93	3.24±0.70	4.58±1.95	4.37±0.58	3.98±0.90
14	3-Methyltricosane	2374	tr	3.19±1.08	7.15±1.76	5.36±1.26	4.81±2.19	4.77±1.08	4.18±1.03
16	2-Methyltetracosane	2464	22.30±24.82	22.76±11.56	13.06±9.31	6.34±1.38	7.65±4.12	7.87±1.46	7.17±2.01
20	9+11-Methylpentacosane	2536	31.70±44.99	30.31±18.40	19.89±13.24	10.63±1.90	17.14±7.75	17.94±2.32	17.56±4.49
21	7-Methylpentacosane	2539	tr	3.19±1.47	3.63±0.91	3.82±1.77	5.14±2.78	3.24±0.91	3.28±1.04
22	5-Methylpentacosane	2549	tr	tr	tr	tr	4.35±2.52	2.49±0.96	3.48±1.03
23	3-Methylpentacosane	2574	5.45±6.96	4.44±2.04	5.87±1.99	7.69±1.25	7.27±3.24	6.41±0.79	6.14±1.57
25	12,22-Dimethylhexacosane[1], 12,20-Dimethylhexacosane[1]	2665	15.26±22.60	12.44±6.84	9.61±6.20	5.58±1.39	9.86±4.40	11.84±1.56	13.45±2.92
29	9+11-Methylheptacosane	2735	tr	tr	2.61±0.96	10.56±2.76	6.31±2.72	4.31±0.63	4.22±1.18
30	3-Methylheptacosane	2774	tr	tr	tr	tr	tr	tr	tr
32	2-Methyloctacosane	2871	5.98±4.83	2.36±0.86	3.56±2.47	3.67±0.99	8.98±3.96	11.69±1.06	12.46±2.63
36	11+13-Methylnonacosane	2937	6.02±5.39	2.99±1.35	4.27±2.98	3.82±1.08	tr	4.38±0.51	4.81±2.14
37	7-Methylnonacosane	2948	tr	tr	tr	3.31±1.80	tr	tr	tr

38	5-Methylnonacosane	2957	tr	tr	tr	3.39±1.12	0.10±0.32	tr	tr
39	Dimethylnonacosane	2966	tr	tr	tr	tr	tr	tr	tr
40	3-Methylnonacosane	2978	tr	tr	tr	9.62±2.57	tr	tr	tr
46	11+15-Methylhentriacontane	3131	tr	tr	4.50±5.66	4.26±1.97	4.88±2.35	4.16±0.49	4.44±1.28
47	3-Methylhentriacontane	3175	tr	tr	tr	5.82±1.96	tr	tr	tr

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			Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Peak	**Peak**	**Kovats**	***n*=10**	***n*=10**	***n*=10**	***n*=10**	***n*=10**	***n*=10**	***n*=10**
no.	**Identification**	**iu**	**%**	**%**	**%**	**%**	**%**	**%**	**%**
5	3-Methylheneicosane	2172	2.97±1.13	2.87±1.03	2.83±0.43	3.92±0.89	5.54±1.93	7.10±2.82	4.55±1.97
7	2-Methyldocosane	2264	tr	tr	tr	tr	tr	tr	tr
11	9+11-Methyltricosane	2339	6.93±1.36	5.67±2.14	7.12±2.12	9.19±1.14	10.11±4.36	10.66±7.84	9.86±10.06
12	7-Methyltricosane	2343	3.33±0.62	2.57±0.92	3.11±0.67	4.48±0.77	4.89±1.95	tr	tr
13	5-Methyltricosane	2348	4.01±0.83	3.68±1.19	3.82±0.88	5.21±0.84	6.37±2.18	6.12±3.13	7.51±10.51
14	3-Methyltricosane	2374	4.05±1.03	3.90±1.41	4.00±1.00	4.83±0.86	4.97±2.17	5.32±2.00	6.88±10.70
16	2-Methyltetracosane	2464	6.76±1.48	6.25±2.17	7.86±6.63	6.19±1.05	5.32±2.40	9.71±6.52	14.59±9.64
20	9+11-Methylpentacosane	2536	16.23±2.94	14.32±5.08	18.29±12.81	16.82±1.87	16.33±7.39	18.98±13.12	15.29±9.74
21	7-Methylpentacosane	2539	2.78±0.36	2.08±0.85	3.22±1.22	4.37±1.14	3.75±2.88	tr	tr
22	5-Methylpentacosane	2549	3.12±0.37	2.81±1.04	3.66±1.12	4.60±1.20	4.39±3.29	tr	tr
23	3-Methylpentacosane	2574	5.86±0.74	5.55±1.81	5.21±2.07	5.01±0.95	4.09±1.22	tr	tr
25	12,22-Dimethylhexacosane[1], 12,20-	2665	13.96±2.49	14.18±4.56	14.66±7.08	11.48±1.12	10.57±4.14	14.00±8.26	13.51±9.57

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	Dimethylhexacosane[1]								
29	9+11-Methylheptacosane	2735	4.13±0.64	4.58±1.12	4.32±0.96	3.71±0.60	tr	tr	tr
30	3-Methylheptacosane	2774	tr	2.05±0.63	tr	tr	tr	tr	tr
32	2-Methyloctacosane	2871	12.95±2.97	13.66±3.43	9.00±1.24	7.00±0.79	6.17±2.60	6.81±4.27	7.98±10.56
36	11+13-Methylnonacosane	2937	6.71±1.19	9.36±2.47	8.13±2.00	7.53±0.90	8.16±2.94	8.91±5.54	9.83±10.14
37	7-Methylnonacosane	2948	0.77±0.31	tr	tr	tr	tr	tr	tr
38	5-Methylnonacosane	2957	1.09±0.49	tr	tr	tr	tr	tr	tr
39	Dimethylnonacosane	2966	tr	2.05±0.79	tr	tr	tr	tr	tr
40	3-Methylnonacosane	2978	tr	tr	tr	tr	tr	tr	tr
46	11+15-Methylhentriacontane	3131	4.37±1.81	4.42±1.86	4.80±0.90	5.65±0.73	9.36±3.59	12.38±5.99	10.00±10.05
47	3-Methylhentriacontane	3175	tr	tr	tr	tr	tr	tr	tr

PCA analysis was preliminarily carried out including all three classes of hydrocarbons (*n*-alkanes, alkenes and methyl branched alkanes), but as with *C. vicina*, ageing could not be determined from the plot due to substantial scatter. A dataset containing just the methyl branched compounds provided the best PCA plot, shown in Figure 4.

As with *C. vicina*, PCA was carried out using the first 6 principal components which described 97.2% of the variation within the data sets with the first four principal components, comprising 73.3%, 8.6%, 6.6% and 4.1% respectively. PC3 and PC4 were used to plot the relevant PCA scores (Figure 4).

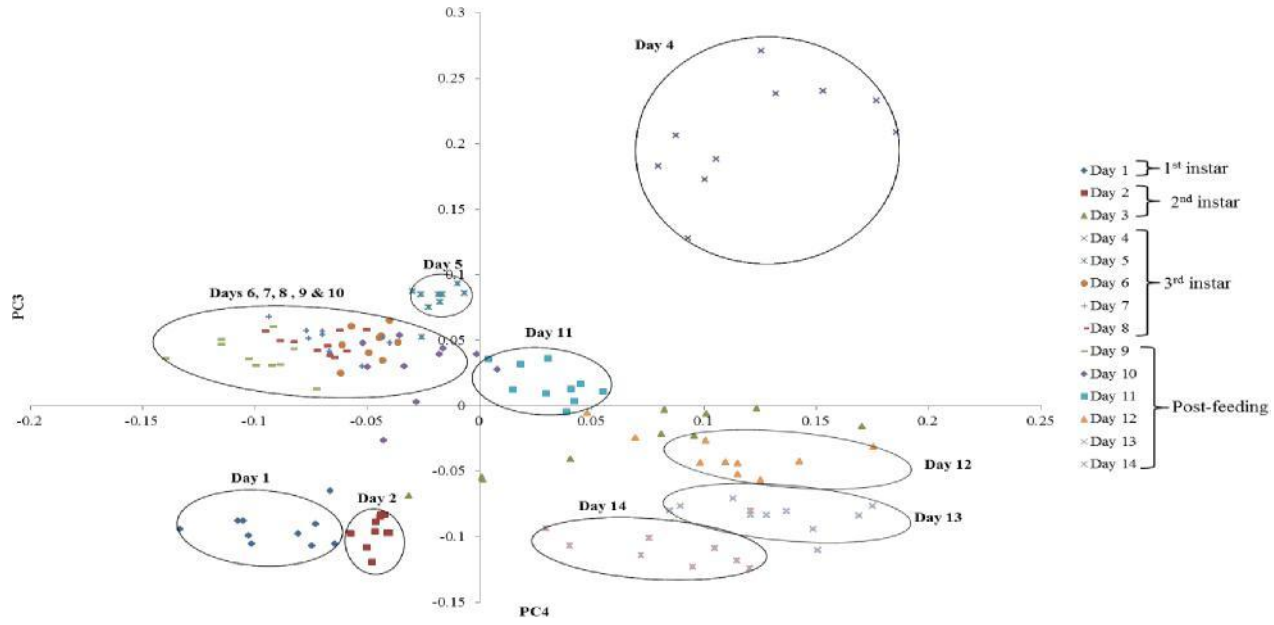

Figure 4: PCA plot showing PC4 against PC3 for *C. vomitoria* larvae using methyl branched alkanes only, with clustering days circled to catch the majority of that days points.

The PCA plot in Figure 4 gives significantly enhanced clustering within the PCA plot, allowing for ageing to be established to a much higher degree of accuracy, with the exception of the third instar which clusters days 6 to 10 in the same group. There are still some outliers that must not be overlooked, for example day 1. However, the post-feeding stage can be aged to the individual day, which is highly advantageous as this is usually a problematic stage to

age using current ageing techniques.

The compound that exhibited the largest PCA loading and therefore was the most significant in the changes seen in the PCA plot was 11+9-Methylpentacosane.

Neural network analysis

Table 6 shows the generalization performance of trained SOMs for each dataset below with the average test performance and standard deviation shown in parenthesis. As the table shows, the best test performance achieved by the SOM for both *C. vicina* and *C. vomitoria* was when the average of five samples was used as the test data, where results are improved by 11 and 17% respectively. This is to be expected as a result of the variation between hydrocarbon profile samples of individual larvae and was shown to be the case when classifying *L. sericata* previously [16]. The majority of the test errors given by each SOM are likely to be a result of similarities between hydrocarbon profiles of larvae which are of a similar age as was shown by the PCA analysis in Figures 3 and 4 where certain ages were within the same clusters. For example, when testing using the average of the remaining five hydrocarbon profiles the majority of errors were misclassifications of +/- 1 day, as shown by the confusion matrices in Table 7.

Table 6: The overall test performance of each SOM when classifying the larvae of *C.vicina* and *C. vomitoria* hydrocarbon profiles.

Test approach	**% correct (SD)**	
	C. vicina	***C. vomitoria***
Average of five samples	89.09 (15.04)	87.86 (16.26)
Individual samples	78.4 (19.58)	70.714 (20.19)

Table 7: Confusion matrices showing the performance of each SOM when classifying for each fold of cross-validation as well as the overall classification performance for each day when tested using the average of the remaining five input patterns of *C.vicina* (top) and *C. vomitoria* (bottom) hydrocarbon profiles.

SOM classification	Input pattern tested										
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11
D1	9	1									
D2	1	9									
D3			10								
D4				9							
D5				1	10						
D6						9					
D7						1	10				
D8								10			
D9									5	1	
D10									4	7	
D11									1	2	10
% correct	90	90	100	90	100	90	100	100	50	70	100

SOM classification	Input pattern tested													
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12	D13	D14
D1	8													
D2	2	10												
D3			9											
D4				10										
D5					10									
D6						10	1							
D7							6	2						
D8							3	5						
D9								1	10					
D10								2		10				
D11											9	2		
D12											1	8		
D13			1										10	2
D14														8
% correct	80	100	90	100	100	100	60	50	100	100	90	80	100	80

Discussion

Roux and co-workers [14] examined the cuticular hydrocarbons of three forensically important blowflies (*C. vicina*, *C. vomitoria* and *P. terraenovae*). They examined the ontogenetic study of these three species from egg through to 8 day old adult flies. Similar observations were noted in comparison to the larvae results presented in this study. The chemical profiles of larvae and post-feeding larvae contain short chain hydrocarbons which evolve into long chain compounds in the pupae and adult flies. The methyl branched alkanes

were also seen to be more abundant in the immature stages of the larvae, with a substantial decrease as they became post-feeding [12].

The PCA plots (Figures 3 & 4) show the potential to age the larvae down to the day, with the exception of the first and second instar larvae (days 1 and 2) and late post-feeding larvae (days 9 to 11) for *C. vicina* and mid-aged third instar larvae (days 6-10) for *C. vomitoria*, which cluster together into one group within the plot.

The very tight clustering observed in *C. vicina* days 1 and 2 are maybe due to the eight methyl branched compounds these two days alone share. Day 3 has a few principal components within its clustering region from day 4, indicating some of the extracted larvae were developing at a slightly faster rate than others. The clusters seen in the PCA plot (Figure 3) form a systematic sequence that tracks the chemical changes. Starting from days 1 and 2 on the bottom left, the changes can be followed in a largely clockwise direction. The large jump from days 1 and 2 to day 3 corresponds to the chemical change going from the 1st and 2$_{nd}$ instar to the 3rd instar.

For *C. vomitoria*, the late post feeding stage can be aged to the day (Figure 4) with days 11 to 14 all individually clustering. This stage is often extremely difficult to age and there are currently no publications able to age this larval stage accurately. This technique therefore shows very promising results for this particular life stage of *C. vomitoria*.

As the larvae age, the higher boiling point alkanes become more abundant. The heavier long chain hydrocarbons are believed to be involved in waterproofing [35-37] which could explain why the larvae of the *Calliphora* species exhibit an increase in these compounds at a later age. When the larvae become older and gradually move into the post-feeding stage of the life cycle they move away from the source of food and seek a site for pupariation, exposing them to a drier environment. Therefore they have a greater need for extra waterproofing compared to their younger age where they are usually at least partly submerged within their food source which is warm

and moist. The display of higher boiling point *n*-alkanes mixed in with alkenes and methyl branched hydrocarbons has also been linked to flexibility of the cuticle [38]. To help flexibility in the larvae's cuticle it will need a composition of methyl branched alkanes and alkenes, which have lower melting points compared to the straight chain alkanes. Further analysis using ANNs allows for the automated classification of newly collected samples without the need for analysis of principal component plots while maintaining a high level of performance. A high performance was achieved for both *C. vicina* and *C. vomitoria* where 89 and 87% accuracy was achieved respectively when testing using an average of five hydrocarbon profiles. Further analysis of the performance of each SOM for both species using confusion matrices showed that a large proportion of test error was caused by the misclassification of larvae by +/- 1 day. This was true for all test cases when classifying *C. vicina* and for all but 3 cases when classifying *C. vomitoria*. Larger datasets for training and testing are likely to improve these results further.

Conclusion

This study has successfully performed preliminary CHC analysis with the aid of statistical analysis to determine the age of larvae from two forensically important *Calliphora* species under controlled laboratory conditions.

Early results show great potential to utilise this technique and to develop it into a highly useful automated ageing tool using principal component analysis and artificial neural networks where test accuracy scores of 78% and 70% were obtained for *C. vicina and C. vomitoria* respectively when using individual hydrocarbon profiles. This performance was improved when testing using an average of the remaining five hydrocarbon profiles (i.e. those that were not used to train the neural network) to provide 89% and 87% for *C. vicina and C. vomitoria* respectively. The majority of errors given by the trained networks were

misclassifications of larvae by +/- 1 day. Further analysis and larger datasets are required to verify these results and assess the suitability of such algorithms for the automated classification of hydrocarbon profiles for accurate $_{PMImin}$ estimations.

Further work is needed to develop this technique. Results presented in this study were executed in a controlled laboratory environment; however experiments need to be carried out in the field to look at the effects that weathering may have on the stability of the hydrocarbons. On-going method development will test if practical implications will be an issue for hydrocarbon analysis, for example, pooled samples collected from the crime scene of young and old larvae.

This study has shown the great potential of hydrocarbon use coupled with statistical techniques for accurate and automated PMI_{min} estimations. This work should now be repeated and validated in the field to test the stability of the hydrocarbons as well as the practicalities of the proposed techniques shown within this paper.

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***Revision Notes (Response to Reviewers)**

Comments to the editor

Dear Szymon,

On behalf of the authors, we would like to thank you for reviewing our paper revisions for publication in FSI. We have read the comments and suggestions and we would be grateful if you could reconsider our paper now that it has further benefitted from the recommendations and amendments that we detail below.

Reviewer(s)' Comments to Author: Reviewer 2

1. *The authors have accepted this comment in regard to temperature as this is indeed an important factor and although hydrocarbons are stable, temperature and environmental factors will of course always be important and will need to be factored in. Page 2, line 42-44.*

2. **Title: Although it would be two words longer, perhaps a more precise title could be considered, i.e., "Age estimation of Calliphora (Diptera: Calliphoridae) larvae using cuticular hydrocarbon analysis and Artificial Neural Networks".**
   Amendment changed
3. **Lines 30-31: Place a full stop after "[12]" and get rid of the line break between sentences.**
   Amended
4. **Lines 67-68: Place a full stop after "SOM training" and get rid of the line break between sentences.**
   Amended
5. **Line 95: Delete "enough" from "sufficient enough".**
   Amended
6. **Line 97: Change "20 first instar" to "20-30 first instar" (see numbers of C. vomitoria 1st instar in Table 1).**
   Amended
7. **Lines 252-253: Clarify sentence by change to, "…27 hydrocarbon peaks were used for PCA analysis, of which 76 % were methyl branched alkanes and 24 % were alkenes.".**
   Amended
8. **Line 305: For clarity, suggest adding to the end of the Figure 4 legend, "…with clustering days circled to catch the majority of that days points.".**
   Amended
9. **Lines 308-309: Day 3 is given as an example of outliers that must not be overlooked, but Day 3 is not a good example because no cluster was drawn and all points are outliers! Better to use as an example of outliers a day for which a cluster is drawn for which there is an outlier, e.g. Day 1.**
   Amended
10. **Line 345: For clarity (and similarity to the way C. vicina is discussed) I suggest changing sentence to, "…for C. vicina and mid-aged third instar larvae (days 6-10) for C. vomitoria, which…".**
    Amended