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

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

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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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6 using cuticular hydrocarbon analysis and Artificial Neural
7 Networks

8 **Abstract**

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

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

21 **Introduction**

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

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

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

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

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

41 CHC analysis has the potential to be able to age larvae as they develop through the larval life
42 stages with a good degree of accuracy [1, 13-14, 24]. These characteristics therefore hold a lot of
43 potential in the field of forensic entomology for PMI estimations. CHCs have the advantage of
44 determining the age to the day, rather than to the life stage and compliment the current techniques
45 used in the field. For more information on CHCs, the reader is referred to the following references
46 [25-27].

47

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

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

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

64
65 Briefly, the unsupervised training of a SOM involves the presentation of every input pattern to the
66 input layer where the incoming weighted connections of the output neuron which best matches the
67 input pattern (known as the winning neuron) are modified. This change in weights results in the
68 formation of clusters within the output layer where similar input patterns cluster in close proximity
69 in the output layer. Readers are referred to [32] and [33] for further details on SOM training.

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

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

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

81 **Material and Materials**

82 *Insect materials*

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

94

95 *Sample Preparation*

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

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

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

113

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

114 *Chemical analysis of extracts*

115 Chemical analysis of all extracts was carried out on an Agilent Technologies 6890N Network GC with a
116 split/splitless injector at 250 °C, a Restek Rxi-1MS capillary column (30m x 0.25 mm ID, 0.25µm film
117 thickness) and coupled to an Agilent 5973 Network Mass Selective Detector. The GC was coupled to a
118 computer and data processed with Agilent Chemstation software. Elution was carried out with helium at
119 1mL/min. The oven temperature was programmed to be held at 50 °C for 2 minutes then ramped to 200
120 °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
121 where it was held for 2 minutes. The mass spectrometer was operated in Electron Ionisation mode at 70
122 eV, scanning from 40 – 500 amu at 1.5 scans s⁻¹. Hydrocarbons were identified using a library search
123 (NIST08), the diagnostic fragmented ions and the Kovats indices.

124 *Statistical analysis:*

125 *Principal Component Analysis*

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

131 *Artificial Neural Networks*

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

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

139 where v_n is the normalised data, v is the original data, $\max(v)$ and $\min(v)$ are the maximum
140 and minimum data values respectively and U_{lim} and L_{lim} are the desired upper and lower limits
141 of the normalised data which in this study are set to +1 and -1 respectively. Training data
142 was presented to the SOM by averaging five hydrocarbon profiles for both species' larvae
143 and adult flies. Testing the generalization performance of the SOM was estimated using
144 two approaches:

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

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

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

155 **Results**

156 *GC-MS analysis:*

157 *C. vicina*

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

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

170 **Table 2** List of all compounds extracted from the larvae of *C. vicina* and their calculated Kovats Indices to
 171 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	Eicosene¹	1990
2	Eicosane	2000
3	Heneicosane	2100
4	Docosene¹	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	Pentacosene¹	2471
13	Pentacosene¹	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-Dimethylhexacosane² + Heptacosene¹	2666
22	Heptacosene¹	2676
23	Heptacosene¹	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	Triacotane	3000
37	2-Methyltricotane	3067
38	2,6/2,8/2,10-Dimethyltriacontane²	3097

172	39	Hentriacontane	3100
173	40	Trtriacontane	3200

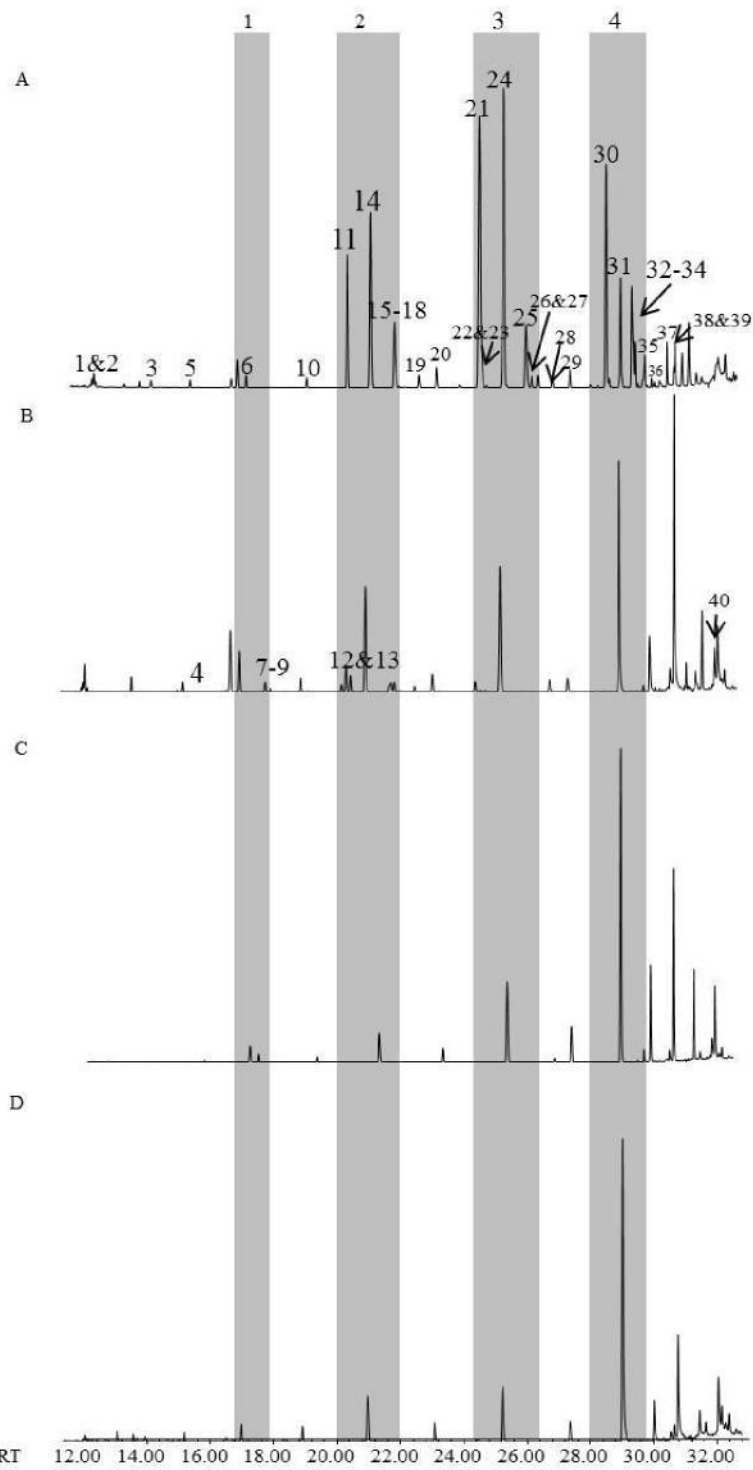
174 ¹Double bond position assumed but not assigned to specific peaks

175 ²Tentative Identification based on calculated Kovats Indices values and match with NIST08 Library

176 database

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

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



187

188

189

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

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

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

200 *GC-MS analysis: C. vomitoria*

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

205 **Table 3:** List of all compounds from C20:H extracted from the larvae of *C. vomitoria* and their Kovats
 206 Indices to aid identification. Compounds in bold were used for subsequent PCA (peak numbers refer to
 207 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

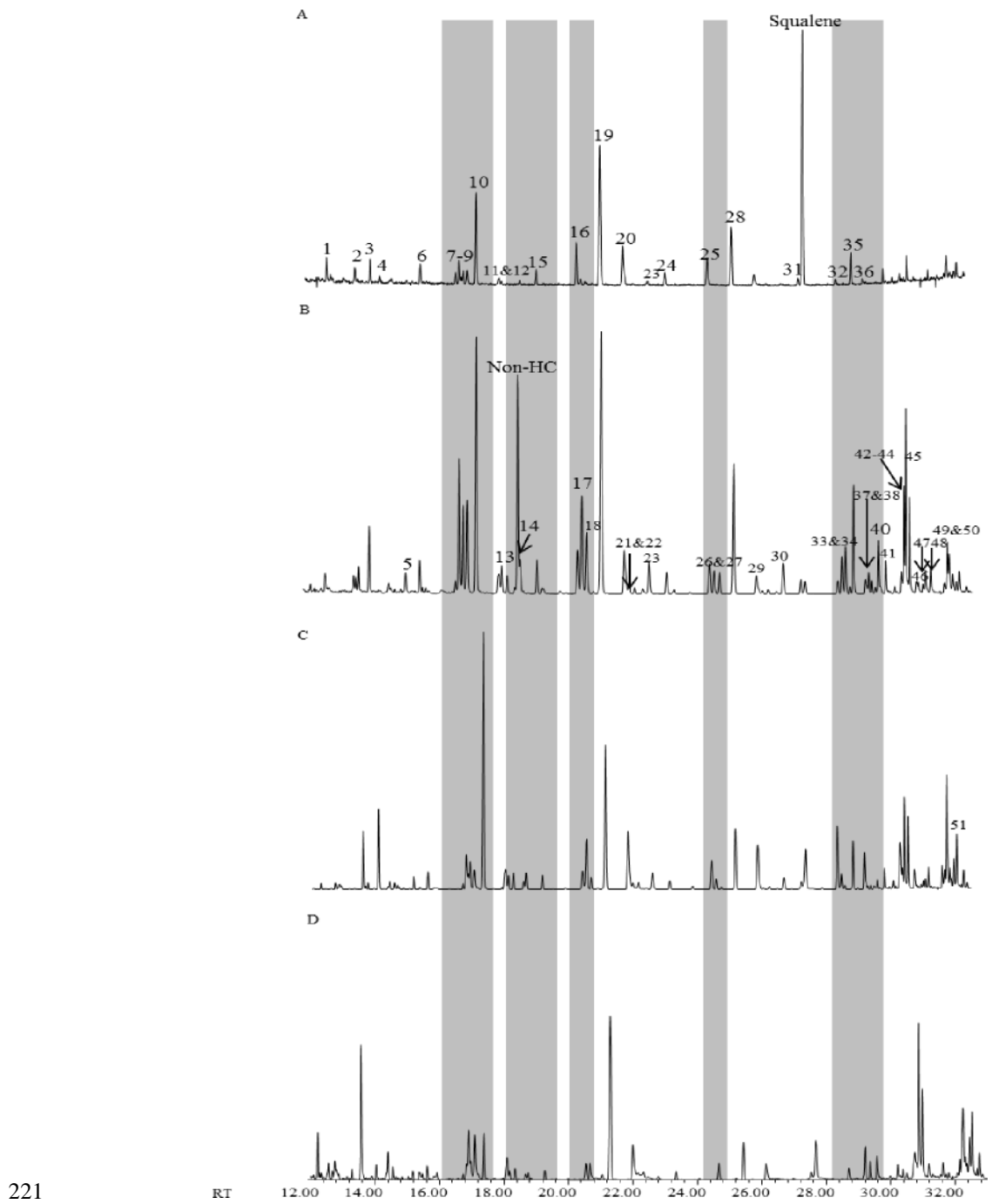
38	5-Methylnonacosane	2957	
39	Dimethylnonacosane	2966	
40	3-Methylnonacosane	2978	
41	Tricontane	3000	
42	Cholesterol + Hentriacontene ¹	3070	
43	Hentriacontene ¹	3077	
44	Hentriacontene ¹	3085	
45	Hentriacontane	3100	
46	11+15-Methylhentriacontane	3131	
47	3-Methylhentriacontane	3175	
48	Dotriacontane	3200	
49	Tritriacontene ¹	3263	
50	Tritriacontene ¹	3276	
208	51	Tritriacontane	3300

209 ¹ Double bond position assumed but not assigned to specific peaks

210 Figure 2 shows the chromatograms of a single sample (Table 1) from days 1, 4, 8 and 13.

211 Chemical distinctions can be made between the different ages from the chromatogram
212 comparison over time. The shaded bars highlight some regions of contrast within the
213 profiles of the different ages.

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



221

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

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

230 2-MeC22:H is only present in the early larval life stages (days 1 to 3) and decreases with
231 age so the presence of this compound could be used to determine the early larval life age
232 (1st and 2nd instar). 7-MeC23:H and 3-MeC23:H (Table 4) are present in a high
233 concentration in day 3. 2-MeC24:H is present in substantial concentrations in days 1 and 2
234 then decrease with age, before an increase is seen in the late post-feeding stage. 9+11-
235 MeC25:H and 2-MeC26:H also appear in a very high concentration in the immature larvae
236 stages (days 1 and 2). Three peaks are absent (below the peak area threshold of 0.5%) from
237 the 1st and 2nd instar larvae (3-MeC21:H, 9+11-MeC27:H and 11+15-MeC31:H) and three
238 compounds are absent from the 1st instar larvae alone (compounds 5-MeC23:H, 3-
239 MeC23:H and 7-MeC25:H). The absence of these compounds from the immature life
240 stages makes them good age indicators.

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

248 *Principal Component Analysis:*

249 *C. vicina:*

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

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

259 **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
 260 percentage standard deviation for each day and the calculated Kovats Indices to aid identification.
 261

Peak number	Peak Identification	Kovats iu	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11
			<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =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

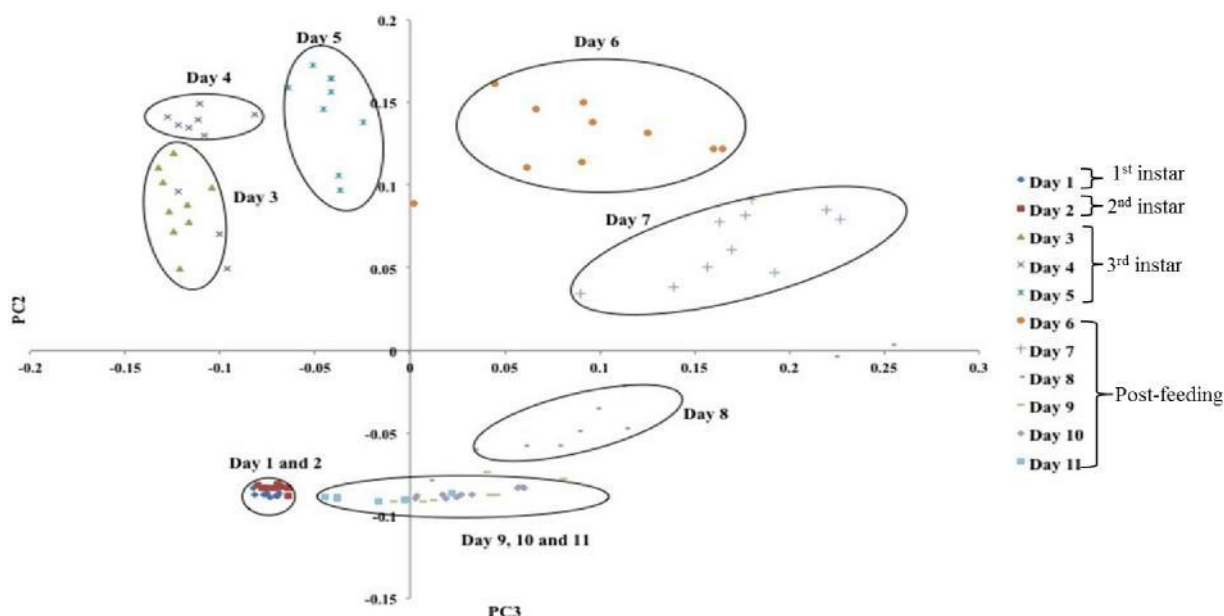
28	3-Methylheptacosane	2775	1.03±0.86	2.08±1.44	6.15±3.65	8.10±3.06	8.04±4.85	11.47±4.61	17.47±1.83	8.76±3.51	tr	tr	tr
30	2-Methyloctacosane	2871	8.53±7.04	11.28±16.23	3.74±2.65	4.14±2.32	2.91±1.67	4.46±1.21	4.95±1.35	tr	tr	tr	tr
32	11+13-Methylnonacosane	2936	2.75±2.87	3.78±5.39	tr	tr	tr	tr	tr	tr	tr	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
262	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

263 ¹Double bond position assumed but not assigned to specific peaks

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

265 tr = Trace amounts detected <0.5%

266 PCA was carried out using the first 6 principal components which described 96.9% of
267 the variation within the data set with the first three principal components, comprising
268 60.5%, 20.3% and 8.9% respectively.



269

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

272 Figure 3 shows the PCA plot of PC3 vs PC2 for data gathered from day 1 to day 11 of
273 larvae extractions of *C. vicina*. There are eight clusters within the plot allowing for the
274 larvae to be aged down to the day with the exception of days 1 and 2 and the late post-
275 feeding stage (days 9 to 11). The clustering groups follow a systematic pattern
276 clockwise around the plot. Although day 1 has a single compound specific to that day,
277 day 1 and day 2 have eight compounds detectable only in those two life stages (1st and
278 2nd instar), hence the likely reasoning for these two stages clustering together in the
279 PCA plot. There is a substantial change within the PCA plot from day 2 to day 3,
280 which represents the transition between the 2nd and 3rd instar.

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

287 *C. vomitoria*:

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

292 **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,
 293 the percentage standard deviation for each day and the calculated Kovats Indices to aid identification
 294

Peak no.	Peak Identification	Kovats iu	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
			<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =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

295

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

Peak no.	Peak Identification	Kovats iu	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
			<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %	<i>n</i> =10 %
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

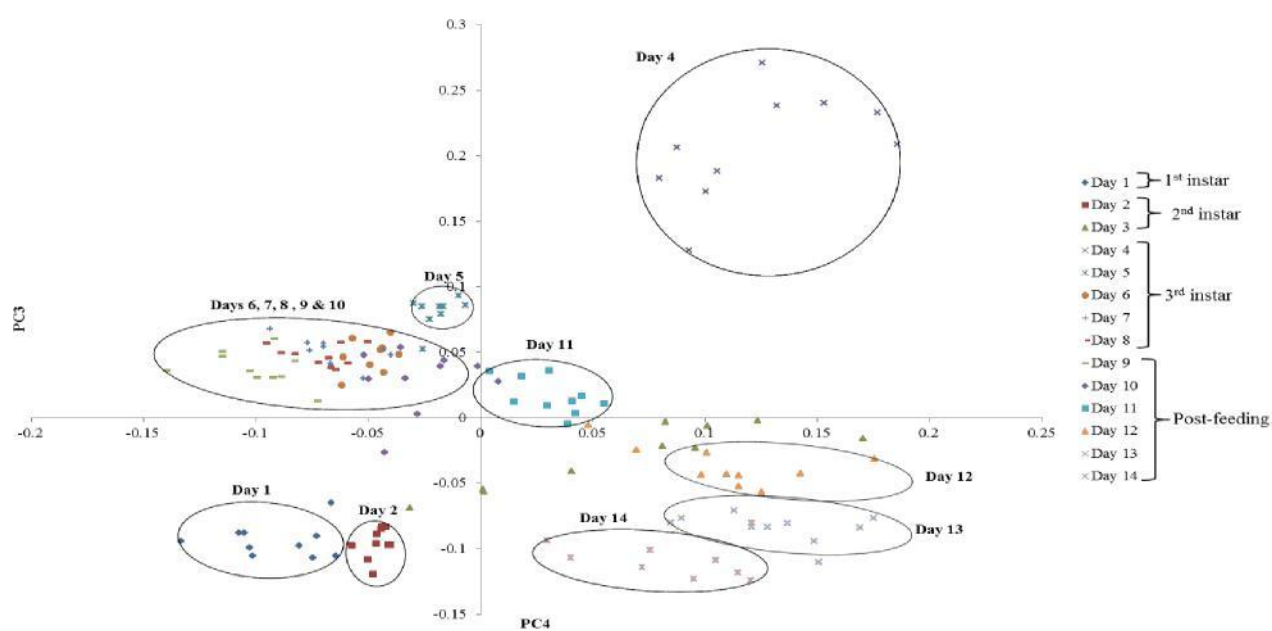
296

Dimethylhexacosane¹

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

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

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



305
306 **Figure 4:** PCA plot showing PC4 against PC3 for *C. vomitoria* larvae using methyl branched alkanes only, with
307 clustering days circled to catch the majority of that days points.

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

313 age using current ageing techniques.

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

316 *Neural network analysis*

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

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

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

331 **Table 7:** Confusion matrices showing the performance of each SOM when classifying for each fold of cross-
 332 validation as well as the overall classification performance for each day when tested using the average of the
 333 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

334

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

335

336 Discussion

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

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

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

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

357 For *C. vomitoria*, the late post feeding stage can be aged to the day (Figure 4) with days 11 to
358 14 all individually clustering. This stage is often extremely difficult to age and there are
359 currently no publications able to age this larval stage accurately. This technique therefore
360 shows very promising results for this particular life stage of *C. vomitoria*.

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

369 and moist. The display of higher boiling point *n*-alkanes mixed in with alkenes and methyl
370 branched hydrocarbons has also been linked to flexibility of the cuticle [38]. To help
371 flexibility in the larvae's cuticle it will need a composition of methyl branched alkanes and
372 alkenes, which have lower melting points compared to the straight chain alkanes. Further
373 analysis using ANNs allows for the automated classification of newly collected samples
374 without the need for analysis of principal component plots while maintaining a high level of
375 performance. A high performance was achieved for both *C. vicina* and *C. vomitoria* where
376 89 and 87% accuracy was achieved respectively when testing using an average of five
377 hydrocarbon profiles. Further analysis of the performance of each SOM for both species
378 using confusion matrices showed that a large proportion of test error was caused by the
379 misclassification of larvae by +/- 1 day. This was true for all test cases when classifying *C.*
380 *vicina* and for all but 3 cases when classifying *C. vomitoria*. Larger datasets for training and
381 testing are likely to improve these results further.

382 **Conclusion**

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

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

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

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

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

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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