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Effect of age on cuticular hydrocarbon profiles in adult *Chrysomya putoria* (Diptera: Calliphoridae)

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Abstract

A species-specific complex mixture of highly stable cuticular hydrocarbons (CHCs) covers the external surface of all insects. Components can be readily analyzed by gas chromatography coupled to mass spectrometry (GC-MS) to obtain a cuticular hydrocarbon profile, which may be used as an additional tool for the taxonomic differentiation of insect species and also for the determination of the age and sex of adult and immature forms. We used GC-MS to identify and quantify the CHCs of female and male *Chrysomya putoria* (Wiedemann, 1818) (Diptera: Calliphoridae) from one to five days old. CHCs ranged from C21 to C35 for females and from C21 to C37 in males. Major compounds were the same for both sexes and were 2-MeC28, C29:1, *n*-C29, 15-,13-MeC29, 2-MeC30, C31:1, *n*-C31 and 15-,13-MeC31. The relative abundance of each component, however, varied with age. Cluster Analysis using Bray-Curtis measure for abundance showed that cuticular hydrocarbon profiles are a strong and useful tool for the determination of age in adult *C. putoria*.

Graphical abstract

Keywords

Cuticular hydrocarbons; Calliphoridae; GC-MS; adults; age; sex

1. Introduction

All insects are covered by a layer of species-specific cuticular lipid that often consists of a complex mixture of hydrocarbons (CHCs) whose main roles are to limit water loss, and in many species, to function in chemical communication. The cuticular hydrocarbons include *n*-alkanes, terminally and internally branched mono and multimethyl alkanes, and alkenes [1-10].

Although molecular techniques can be used for the separation of species, DNA and proteins are usually decomposed in insects used for forensic analysis, and are often not useful [11]. CHCs have been used for species differentiation of some insects including parasitic wasps [12], phlebotomines [13,14], anophelines [15,16], culicids [17], triatomines [18,19] and the forensically important Diptera Calliphoridae [20-27] and Sarcophagidae [28].

A number of studies show that the CHC profiles vary according to the age and sex of the insects [17,21,23,24,27,29-43]. These differences may be related to female attractiveness to males and play key roles in reproduction in some species [29,31].

There are relatively few papers on hydrocarbon profiles for necrophagous flies (Diptera Calliphoridae and Sarcophagidae) [20-28]. These insects, including *Chrysomya putoria*, are pioneers in cadaver colonization and play an important role in decomposition. The determination of the late post mortem interval (PMI) is based on the age of larvae and adults and on species succession [30].

The aim of this study was to determine if GC-MS analyses of CHCs could be used to identify the sex and determine the age of one to five day old females and males of the Calliphoridae *C. putoria*.

2. Methodology

2.1. Collection and maintenance of the insects

Colonies of *C. putoria* were established and maintained in the Laboratory of Medical and Forensic Entomology, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil. The insects were placed in cubic cages (30×30×30cm) made of a wooden frame closed with nylon fabric. One of the sides was closed with a sleeve-like fabric to facilitate changes of water and food and to avoid the escape of the flies during these proceedings. The eggs were transferred to a new diet (liver) where they hatched and the larvae developed. Liver was divided in three equal parts (250 g) and offered to the larvae of all four species. After the larvae abandoned the liver, they were individually weighed and transferred to glass tubes and maintained under controlled conditions. One fourth of the test tubes were filled with vermiculite and closed with hydrophobic cotton plugs for the pupation, emergence of the adults and observation of morphological alterations. After the adult emergence they were stored at -20 °C before hydrocarbon extraction.

The colonies were kept under laboratory conditions, in a climatic chamber with 27 ± 1 °C, $60\% \pm 10\%$ Relative Humidity and a 12 hour photoperiod (12 hours light / 12 hours dark) [44]. The F1 was used for the identification of the species using a dichotomous key for Brazilian Calliphoridae [45]. The adults of the F2 were collected daily from day one to day five for cuticular hydrocarbon extraction.

2.2. Cuticular Hydrocarbon (CHC) Extraction

Extraction of CHCs was performed in the Department of Biochemistry & Molecular Biology, University of Nevada, Reno, NV, USA. Thirty (three groups of 10 each) one to five day old females and males of *C. putoria* were extracted with hexane as previously described [28]. After the extraction, the CHCs were concentrated under a stream of nitrogen. The extract was resuspended in 10 µL of redistilled hexane before GC-MS analysis.

2.3. GC-MS analysis

Aliquots (1 µL) were analyzed by a Thermo-Finnigan Trace GC with Polaris Q Mass Spectrometer (GC-MS) in the Proteomics Center of Nevada, UNR, Reno, NV, USA, as previously described [28]. Helium was the carrier gas.

The GC-MS analyses yielded qualitative results and were used to identify components. CHCs with chain lengths of 21 carbons or more were present and used for data analyses. Triplicate analyses were made for each age group of both sexes. The analyzed peaks were numbered according to their retention times. The relative abundance was calculated by computing the area of each peak, producing a percentage of the total peak area of all components in the sample. Only peaks with a relative abundance of 0.1% or more were used in the analyses.

The identification of CHCs from electron impact (EI) mass spectra was as described [3,46]. The positions of the double bonds in the alkenes were not determined due to small sample size. In some peaks two or more isomers eluted together and in those cases the relative abundance could not be individualized for each compound. The nomenclature used to list hydrocarbons in the tables was Cxx to describe the total number of carbons in the linear chain of the compound; the location of methyl groups is indicated by x-Me for monomethylalkanes and x,y-Dime for dimethylalkanes when one or two methyl groups are located in the molecule, respectively. For alkenes the nomenclature was Cxx:z with z indicating the number of double bonds in the chain.

2.4. Statistical Analysis

In order to determine if using hydrocarbon profiles allows discrimination among one to five day old adult females and males of *C. putoria*, statistical analysis was performed using Spearman Rank Order Correlation (SigmaPlot for Windows version 11.0 Build 11.0.0.77) and Cluster Analysis using Bray-Curtis measure for abundance (PAST software, version 2.17). In the case of Spearman Rank Order Correlation, we used all peaks that had a relative abundance of 0.1% or more. For cluster analysis we used only the major peaks (relative abundance over 5%) of each replicate.

3. Results and Discussion

Qualitative and quantitative comparisons of the CHC profiles from one to five-day-old female and male *C. putoria* are demonstrated in Figures 1 and 2 and Tables 1 and 2. Females had more peaks per day on days one, two and five (ranging from 32 to 41 peaks -days four and one respectively) than males (ranging from 31 to 40 peaks - days five and one respectively). The CHC from females had compounds that ranged from 21 to 35 total carbons, whereas males ranged from 21 to 37 total carbons. The hydrocarbon components from both sexes of *C. putoria* include *n*-alkanes, 2- and 3-methylalkanes, internally branched mono- and dimethylalkane, alkenes and alkadienes. In Tables 1 and 2, peak number 45 represents a different dimethylalkanes depending on the age of the fly. The range of chain lengths for the CHCs of *C. putoria* are similar to those of other Diptera. Mosquitoes tend to have CHCs that include somewhat shorter chain length components than other insects. *Aedes aegypti* (Linnaeus, 1762) CHCs range in chain length from C16 to C35 [17] and the CHCs from *Anopheles gambiae* Giles, 1926 ranged from C17 to C47 [34]. CHCs from the fruit fly *Anastrepha fraterculus* (Wiedemann, 1830) varied from C13 to C37 [42]. Moore et al. [24] studied CHCs from blowfly larvae of *Lucilia sericata* (Diptera: Calliphoridae) and obtained chain lengths that varied from C16 to C33.

The positions of the double bonds in the alkenes were not determined due to small sample sizes. This did not interfere with the final interpretation of the results since there were a relatively small number of alkenes in the samples. Most of the alkenes were monoenes and a few dienes were detected (Tables 1 and 2).

There are differences in the relative abundance of CHC components as insects age from day one through day five for both sexes, but these differences are more quantitative than qualitative (Tables 1 and 2). For both sexes some compounds, however, were age exclusive.

Females had less age exclusive compounds (only dimethylalkanes and alkenes) and 13,17-DiMeC29, 6,16-DiMeC30 and 15,21-DiMeC31 for day one had relative abundances over 0.6% (Table 1). Males had more age exclusive compounds but only 6,16-DiMeC30 and 15,19-DiMeC31 for day one were the ones with high relative abundances (over 1%) (Table 2). These results are different from those obtained by Trabalon et al. [29] for *Calliphora vomitoria* (Linnaeus, 1758), which had fewer CHC components in males and a progressive increase in the CHC components in females. These authors also found alkenes only in females of *C. vomitoria*, whereas both sexes of *C. putoria* have alkenes. Horne and Priestman [17] showed that females of the *A. aegypti* had approximately 25% more CHCs than males, which was also observed in *C. vomitoria* [29].

Day one *C. putoria* females had larger amounts of dimethylalkanes (Table 1, Figure 3A) than older insects and more of the longer chain dimethylalkanes and all dimethylalkanes, except 13,17-DiMeC31, were age exclusive (Table 1). For males, however, some dimethylalkanes were age exclusive and others were not (Table 2, Figure 3B). Females had dimethylalkanes than ranged from 29 to 33 carbons in the linear chain while in males these ranged from 27 to 35 carbons in the linear chain (Tables 1 and 2). Horne and Priestman [17] observed that both females and males of *A. aegypti* had only dimethylalkanes with chain lengths above 29.

The CHCs of *C. putoria* contained a mixture of *n*-alkanes, terminally and internally branched monomethylalkanes, dimethylalkanes and unsaturated components (monoenes and dienes) for both sexes at all ages (Fig. 3A and B). Monomethylalkanes were the most abundant for both sexes at all ages followed by the *n*-alkanes (Fig 3A and B). For females two to five days old, terminally branched monomethylalkanes were less abundant than internally branched that gradually increased from day one to four (Fig 3A). Males did not have similar result (Figure 3B). *C. vomitoria* [29] had monomethyl and *n*-alkanes as the most abundant CHCs of the profiles. The buffalo fly *Haematobia exigua* and the horn fly *Haematobia irritans* [35] had *n*-alkanes and monoenes as major compounds. Moore et al. [24], however, detected that larvae of *Lucilia sericata* (Diptera, Calliphoridae) had more *n*-alkanes than the other classes of CHCs.

All comparisons among the age groups for both sexes were highly significantly different ($p < 0.0001$), (Table 3). In the case of females, Trabalon et al. [29] observed that one-day-old females of *C. vomitoria* had a higher amount of CHCs. These results were corroborated by the present study for one-day-old females *C. putoria*. In contrast, two days old males of *C. vomitoria* [29] had a significantly higher number of CHCs, which was different from the present study where one-day-old males of *C. putoria* had more CHCs. Those authors relate the higher amount of CHCs to sexual maturation that occurs at that age (48 hours). Cluster analysis using Bray-Curtis measure of the relative abundances of the major peaks of one to five day old female and male *C. putoria* demonstrated that it is possible to differentiate the ages of both sexes using CHC profiles (Fig 4A and B). The cophenetic correlation was 0.7632 and 0.7137 for females and males, respectively.

Major compounds were the same for all ages of both sexes, varying only in their relative abundances (Fig 5A and B). The major components in both sexes had carbon chains with an odd number of carbons, except for the 2-methylalkanes (2-MeC28 and 2-MeC30). This is

consistent with the known pathways of hydrocarbon formation [47] in which the carboxyl carbon of the precursor fatty acid is removed in the last step [48]. Many dipterans have cuticular hydrocarbon compositions that vary as the insect ages, especially among those species where pheromone components are present as cuticular hydrocarbons. For example, the female housefly has an increase in both (*Z*)-9-tricosene and methylalkanes during days 1-5 [31] and hydrocarbon profiles change in both *A. gambie* [34] and *A. fraterculus* [42] as adults age. A related to age increase or decrease in the relative abundances of some of the CHCs may be observed. The most remarkable increases occurs in *n*-C29 for females and C31:1 for males, and decreases occurs for both sexes in 2-MeC28 (from day two to five for females) and 2-MeC30 for males (Tables 1 and 2, Figure 5). Howard and Pérez-Lachaud [32] analyzed the CHCs of the ectoparasitic wasp *Cephalonomia hyalinipennis* Ashmead, 1893 (Hymenoptera: Bethyridae) and its alternative host *Caulophilus oryzae* (Gyllenhal, 1838) (Coleoptera: Curculionidae). These authors did not observe any effect of the CHC profiles related to sex, age or mating status of the wasps and did not find any significant difference between females and males of its host. It is not known whether the hydrocarbons of *C. putoria* function in chemical communication.

The most abundant *n*-alkanes found in the profiles of females and males of all age groups had from 27 to 31 carbons (Fig 6A and B). There were traces of shorter chain (21 to 26 carbons) *n*-alkanes, as well as the *n*-C32, but *n*-C22 and *n*-C24 were not detected. For females of *C. putoria*, *n*-C27, *n*-C29 and *n*-C31 increased with age, but this did not occur for males. Larvae of *L. sericata* [24] had an increase with age of *n*-C29, *n*-C31 and *n*-C33. Larvae of *Aldrichina graham* (Diptera: Calliphoridae) [27] also had an increase with age of *n*-C25, *n*-C27 and *n*-C31.

4. Self-critique

We made the decision in using only three replicates and not five or more due to the difficulty in the collection and maintenance of a laboratory colony of *C. putoria*. Shortly after the three replicates for each age were separated, the colony was lost due to unrelated facts. The variation in the abundances for the three replicates for both sexes at each age group, however, was small as demonstrated by the standard deviation seen in Figures 5 and 6. Moreover, the comparisons using Spearman correlation and the cluster analysis clearly corroborates our results.

5. Conclusion

CHC profiles are a robust tool for the identification of sex and age of adult *C. putoria*. This study and brings new information on the CHCs of an important species of Calliphoridae, *C. putoria*, which may be useful in the field of forensic entomology.

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Highlights

- Major compounds were the same for one to five days old adults of *Chrysomya putoria*.
- Cluster analysis of major compounds separates clearly the ages of adults.
- Some cuticular hydrocarbons (CHCs) were age exclusive for both sexes.
- CHC profiles are an interesting tool to separate age and sex of adult insects.

Fig 1. Comparison of the cuticular hydrocarbon gas chromatogram profiles (HCs) from one day old (A), two days old (B), three days old (C), four days old (D) and five days old (E) females of *Chrysomya putoria*. The numbers over the peaks indicate the corresponding peaks in Table 1. Grey shading indicates the area of each peak.

Fig 2.

Comparison of the cuticular hydrocarbon gas chromatogram profiles (HCs) from one day old (A), two days old (B), three days old (C), four days old (D) and five days old (E) males of *Chrysomya putoria*. The numbers over the peaks indicate the corresponding peaks in Table 2. Grey shading indicates the area of each peak.

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Fig. 3. Percentages of each class of hydrocarbons present in the profiles from one to five days old females (**A**) and males (**B**) of *Chrysomya putoria*.

Fig. 4. Cluster Analysis using Bray-Curtis measure of the relative abundances of the major compounds for one to five days old females (**A**) and males (**B**) of *Chrysomya putoria*.

Fig. 5. Mean and standard deviations of the relative abundances (%) of the major peaks from one to five days old females (**A**) and males (**B**) of *Chrysomya putoria*.

Fig. 6. Mean and standard deviations of the relative abundances (%) of *n*-alkanes from one to five days old females (A) and males (B) of *Chrysomya putoria*.

Mean and standard deviations of the relative abundances* of cuticular hydrocarbons (CHCs) from one to five-day-old females of *Chrysomya putoria*.

Table 1

Peak	Hydrocarbon	ECL	1 day	2 days	3 days	4 days	5 days
total of CHCs/day							
1	<i>n</i> -C21	21.00	0.20±0.05	0.16±0.05	0.13±0.10	0.14±0.18	0.13±0.05
2	<i>n</i> -C23	23.00	0.26±0.02	0.21±0.11	nd	nd	0.29±0.15
3	11-MeC23	23.34	0.18±0.05	nd	nd	nd	0.1±0.1
4	3-MeC23	23.71	0.11±0.02	nd	nd	nd	nd
5	C25:2	24.04	nd	nd	nd	0.13±0.15	0.15±0.06
6	C25:1	24.72	0.70±0.13	0.11±0.10	nd	nd	0.11±0.09
7	<i>n</i> -C25	25.00	0.63±0.23	0.32±0.26	0.22±0.14	0.25±0.17	0.66±0.33
8	13-11-MeC25	25.34	2.03±0.90	0.54±0.39	0.47±0.29	0.35±0.14	1.49±0.43
9	3-MeC25	25.72	0.42±0.14	0.16±0.12	0.11±0.06	nd	0.12±0.04
10	<i>n</i> -C26	26.00	0.11±0.00	0.16±0.11	0.14±0.07	0.14±0.08	0.16±0.04
11	13-MeC26	26.32	0.19±0.01	0.12±0.09	0.11±0.08	0.54±0.26	0.15±0.07
12	2-MeC26	26.63	0.32±0.05	0.79±0.47	0.5±0.24	nd	0.39±0.16
13	C27:1	26.74	1.19±0.29	0.87±0.34	1.25±0.20	1.26±0.40	0.92±0.10
14	<i>n</i> -C27	27.00	2.61±0.62	2.86±0.30	3.10±0.79	3.57±0.65	4.11±0.93
15	13-11-MeC27	27.31	3.43±0.90	1.49±0.07	1.70±0.25	1.25±0.52	1.99±0.62
16	7-MeC27	27.49	0.60±0.05	0.27±0.03	0.39±0.09	0.40±0.15	0.22±0.04
17	2-MeC27	27.62	0.42±0.02	0.21±0.13	0.13±0.01	nd	0.14±0.04
18	3-MeC27	27.73	2.04±0.50	1.05±0.08	0.82±0.20	0.63±0.30	0.85±0.30
19	<i>n</i> -C28	28.00	0.54±0.05	0.79±0.61	0.73±0.18	0.74±0.25	0.88±0.34
20	14-13-MeC28	28.30	0.28±0.06	nd	0.15±0.04	0.12±0.07	0.11±0.03
21	2-MeC28	28.64	14.63±1.19	15.19±0.34	9.29±1.62	8.19±0.90	7.31±0.29
22	C29:1	28.74	nd	2.97±0.62	5.17±1.23	6.63±1.08	3.54±1.03
23	<i>n</i> -C29	29.00	10.73±0.35	13.31±1.24	13.40±2.05	17.48±3.93	19.32±2.97
24	15-13-MeC29	29.30	7.69±0.44	5.53±0.89	6.42±1.79	5.36±1.06	4.07±0.43
25	13,17-DiMeC29	29.53	1.30±0.06	nd	nd	nd	nd
26	2-MeC29	29.63	0.89±0.10	0.49±0.11	0.43±0.12	0.22±0.06	0.26±0.10
27	3-MeC29	29.73	1.91±0.14	1.48±0.14	1.30±0.27	1.08±0.38	0.98±0.31

Peak	Hydrocarbon	ECL	1 day	2 days	3 days	4 days	5 days
	total of CHCs/day		41	36	35	32	39
28	<i>n</i> -C30	30.00	0.96±0.15	0.86±0.11	1.02±0.23	0.92±0.33	1.27±0.42
29	15-,14-MeC30	30.29	1.05±0.09	0.29±0.04	0.29±0.09	0.26±0.17	0.25±0.11
30	C31:2	30.47	nd	nd	0.14±0.03	nd	nd
31	2-MeC30	30.65	14.11±2.71	18.18±0.39	11.46±1.36	8.28±1.27	9.03±0.43
32	C31:1	30.78	6.22±1.22	14.34±2.00	21.63±0.27	26.72±4.55	21.80±4.75
33	6,16-DiMeC30	30.92	3.25±1.06	nd	nd	nd	nd
34	<i>n</i> -C31	31.00	4.01±0.21	5.76±0.46	5.85±1.34	5.81±1.11	8.16±1.16
35	15-,13-MeC31	31.29	10.89±1.64	7.01±0.27	8.21±2.09	5.69±1.28	6.49±0.87
38	13,17-DiMeC31	31.51	1.87±0.16	1.02±0.20	0.91±0.51	0.29±0.10	0.48±0.15
37	15,21-DiMeC31	31.64	0.67±0.09	nd	nd	nd	nd
38	3-MeC31	31.74	0.41±0.05	0.27±0.07	0.27±0.13	0.14±0.08	0.16±0.05
39	<i>n</i> -C32	32.00	nd	0.11±0.03	0.18±0.03	0.14±0.07	0.19±0.10
40	15-MeC32	32.25	0.18±0.02	0.20±0.27	nd	nd	nd
41	C33:2	32.52	0.16±0.05	0.43±0.08	0.69±0.12	0.18±0.13	0.34±0.10
42	2-MeC32	32.60	0.42±0.11	0.47±0.10	0.37±0.05	0.19±0.07	0.26±0.12
43	C33:1	32.76	0.99±0.11	1.26±0.14	1.99±0.76	1.90±0.69	2.21±0.64
44	17-,15-MeC33	33.26	0.60±0.05	0.27±0.08	0.39±0.06	0.25±0.14	0.38±0.16
45	13,19-DiMeC33	33.56	0.33±0.11	nd	nd	nd	nd
	11,19-DiMeC33	33.57	nd	nd	nd	nd	0.17±0.21

ECL – equivalent chain length;

* the relative abundance of each peak was calculated in relation to the total peak area; nd – not detected or below 0.1% relative abundance (trace). Bold indicates age exclusive compounds. Peak 45 represents a different dimethylalkane for days 1 and 5.

Mean and standard deviations of the relative abundances* of cuticular hydrocarbons (CHCs) from one to five-day-old males of *Chrysomya putoria*.

Table 2

Peak	Hydrocarbon	ECL	1 day	2 days	3 days	4 days	5 days
total of CHCs/day							
1	<i>n</i> -C21	21.00	0.17±0.03	nd	0.14±0.02	nd	nd
2	<i>n</i> -C23	23.00	0.17±0.02	nd	0.17±0.04	nd	nd
3	11-MeC23	23.34	0.16±0.04	nd	nd	nd	nd
4	C25:2	24.04	0.11±0.03	0.15±0.09	nd	nd	nd
5	C25:1	24.72	0.49±0.09	nd	nd	nd	nd
6	<i>n</i> -C25	25.00	0.49±0.11	0.14±0.02	0.17±0.04	0.12±0.01	0.13±0.05
7	13-,11-MeC25	25.34	1.87±0.30	0.48±0.09	0.30±0.02	0.20±0.03	0.56±0.17
8	3-MeC25	25.72	0.35±0.04	0.16±0.02	nd	nd	nd
9	<i>n</i> -C26	26.00	0.12±0.03	0.11±0.03	0.12±0.02	0.11±0.01	nd
10	13-MeC26	26.32	0.26±0.02	0.17±0.03	nd	0.10±0.02	nd
11	2-MeC26	26.62	0.25±0.03	0.41±0.06	0.35±0.09	0.28±0.06	nd
12	C27:1	26.74	1.20±0.12	1.00±0.11	0.85±0.08	1.00±0.19	0.44±0.02
13	<i>n</i> -C27	27.00	2.43±0.47	2.51±0.56	3.09±0.33	3.43±0.19	2.17±0.88
14	13-MeC27	27.33	3.23±0.47	2.12±0.22	1.37±0.13	2.04±0.16	1.84±0.34
15	7-MeC27	27.49	0.42±0.12	0.85±0.43	0.28±0.01	1.01±0.21	0.39±0.05
16	9,15-DiMeC27	27.57	nd	nd	nd	nd	0.18±0.12
17	2-MeC27	27.63	0.31±0.06	0.19±0.10	nd	0.23±0.08	nd
18	3-MeC27	27.74	1.30±0.10	0.99±0.08	0.90±0.07	1.14±0.17	0.73±0.04
19	<i>n</i> -C28	28.00	0.51±0.37	0.84±0.18	0.79±0.02	1.22±0.07	0.93±0.30
20	14-,13-MeC28	28.29	0.36±0.09	0.29±0.06	0.14±0.01	0.34±0.00	0.21±0.04
21	2-MeC28	28.65	13.38±1.11	11.33±0.74	10.28±2.24	7.63±0.31	5.19±1.59
22	C29:1	28.74	nd	3.89±0.87	3.59±0.36	6.29±0.17	5.27±0.55
23	<i>n</i> -C29	29.00	11.34±2.23	12.11±2.69	16.24±1.50	12.61±0.88	14.04±2.77
24	15-,13-MeC29	29.29	7.27±1.26	9.20±2.30	5.95±0.50	9.17±0.19	9.10±2.36
25	13,17-DiMeC29	29.53	1.45±0.20	1.51±0.45	0.45±0.13	1.48±0.26	0.69±0.21
26	2-MeC29	29.60	nd	nd	0.38±0.09	nd	0.19±0.10
27	3-MeC29	29.72	2.88±0.36	2.41±0.32	1.23±0.09	2.62±0.18	1.17±0.20

Peak	Hydrocarbon	ECL	1 day	2 days	3 days	4 days	5 days
	total of CHCs/day		40	33	36	36	31
28	<i>n</i> -C30	30.00	1.06±0.16	1.00±0.16	1.13±0.10	1.67±0.08	1.45±0.54
29	15-,14-MeC30	30.28	0.91±0.22	0.84±0.16	0.44±0.02	1.21±0.10	0.79±0.10
30	2-MeC30	30.67	16.58±3.83	15.08±3.83	13.79±1.26	8.58±0.51	8.02±1.98
31	C31:1	30.79	5.83±1.86	13.11±2.54	18.36±2.24	16.64±1.26	20.63±3.74
32	6,16-DiMeC30	30.90	4.70±1.08	nd	nd	nd	nd
33	<i>n</i> -C31	31.00	5.48±0.68	5.08±1.96	6.27±0.98	4.83±0.77	6.34±0.61
34	15-,13-MeC31	31.31	8.60±3.01	9.06±2.87	8.92±0.74	9.18±0.45	12.89±2.92
35	13,17-DiMeC31	31.52	2.31±0.48	2.30±0.31	0.94±0.26	1.22±0.11	1.10±0.27
36	15,19-DiMeC31	31.64	1.41±0.27	nd	nd	nd	nd
37	3-MeC31	31.76	nd	nd	0.22±0.08	0.24±0.02	0.19±0.06
38	<i>n</i> -C32	32.00	0.10±0.02	0.12±0.04	0.17±0.02	0.31±0.09	0.32±0.08
39	16-,15-,14-MeC32	32.28	0.12±0.06	nd	nd	0.15±0.04	nd
40	C33:2	32.43	nd	nd	0.12±0.05	nd	nd
41	2-MeC32	32.65	0.57±0.11	0.31±0.10	0.28±0.03	0.33±0.08	0.19±0.09
42	C33:1	32.80	0.39±0.11	0.88±0.17	1.31±0.04	2.03±0.43	1.96±0.31
43	C33:1	32.90	0.40±0.10	nd	nd	nd	nd
44	17-,15-MeC33	33.30	0.41±0.21	0.37±0.13	0.40±0.03	0.89±0.17	0.96±0.14
	13,21-DiMeC33	33.58	nd	nd	0.28±0.06	nd	nd
45	11,21-DiMeC33	33.59	nd	nd	nd	0.65±0.07	0.76±0.23
	11,19-DiMeC33	33.60	0.38±0.07	0.37±0.12	nd	nd	nd
46	13-MeC35	35.30	nd	nd	nd	0.11±0.01	nd
47	13,23-DiMeC35	35.54	nd	nd	nd	0.16±0.00	0.22±0.07

ECL – equivalent chain length;

* the relative abundance of each peak was calculated in relation to the total peak area; nd – not detected or below 0.1% relative abundance (trace). Bold indicates age exclusive compounds, Peak 45 represents a different dimethylalkane for days 1 and 2, day 3 and days 4 and 5.

Table 3

Result of the Spearman Rank Order Correlation (p) comparing the hydrocarbon profiles from one to five days old females and males of *Chrysomya putoria*. Highlighted cell = ♀×♀ results; cells with borders = ♂×♂ results; no markings = ♀×♂ results.

		♀ 2 days	♀ 3 days	♀ 4 days	♀ 5 days	♂ 1 day	♂ 2 days	♂ 3 days	♂ 4 days	♂ 5 days
♀ 1 day	c.c. p value	0.736 2.00E-07	0.689 2.00E-07	0.670 2.00E-07	0.676 2.00E-07	0.835 2.00E-07	0.718 2.00E-07	0.751 2.00E-07	0.682 2.00E-07	0.667 2.00E-07
♀ 2 days	c.c. p value		0.948 2.00E-07	0.895 2.00E-07	0.938 2.00E-07	0.613 1.75E-07	0.816 2.00E-07	0.897 2.00E-07	0.822 2.00E-07	0.781 2.00E-07
♀ 3 days	c.c. p value			0.950 2.00E-07	0.922 2.00E-07	0.602 3.19E-07	0.840 2.00E-07	0.916 2.00E-07	0.849 2.00E-07	0.825 2.00E-07
♀ 4 days	c.c. p value				0.903 2.00E-07	0.637 3.78E-07	0.824 2.00E-07	0.869 2.00E-07	0.836 2.00E-07	0.845 2.00E-07
♀ 5 days	c.c. p value					0.613 1.82E-06	0.793 2.00E-07	0.875 2.00E-07	0.806 2.00E-07	0.810 2.00E-07
♂ 1 day	c.c. p value						0.763 2.00E-07	0.648 1.37E-07	0.623 9.83E-07	0.623 9.77E-07
♂ 2 days	c.c. p value							0.871 2.00E-07	0.881 2.00E-07	0.840 2.00E-07
♂ 3 days	c.c. p value								0.902 2.00E-07	0.875 2.00E-07
♂ 4 days	c.c. p value									0.946 2.00E-07

c.c. = correlation coefficient; all comparisons were significantly different (p < 0.05).